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STUDIES ON "NUCLEOTIDE-*N*-RIBOSIDASE".

By

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(Received for publication, February 19, 1937)

In a previous communication the present writer (1936) demonstrated in animal tissues the existence of an enzyme which disrupted especially the glycosidic linkages of purine ribonucleotides, yielding ribophosphoric ester and free purine bases. This new enzyme, which may be hereafter called ''nucleotide-*N*-ribosidase'', was present in animal tissues always together with nucleosidase, being occasionally accompanied by nucleotidase or purine-deaminases. On the one hand, the enzyme was distributed among various tissues in quite different quantities; on the other hand, the content of the enzyme in any one tissue varied considerably according to the species of animal. The *N*-ribosidase and nucleosidase displayed a close similarity in many respects.

The investigation of which the results are now communicated was designed to acquire more definite information about the *N*-ribosidase.

EXPERIMENTAL.

Ishikawa's technique (1935) was employed for the quantitative determination of the products of enzymic hydrolysis of the substrates. The previous communication is to be referred to also. The substrates employed in the present experiment were guanine ribonucleotide and guanine riboside. The disruption of the glycosidic linkage of guanylic acid was measured from the quantity of organic phosphorus in the mercuric sulphate-filtrate of the solution to be tested; this phosphorus signifies the ribosephosphoric ester formed. Phosphorus was determined by the spectrophotometric method of Teorell (1931). The subsequent hydrolysis of the ester once formed was practically excluded, since the enzyme samples used in the present experiments contained phosphatase only in a very small quantity. The hydrolysis of guanosine was estimated by determining the reducing power of the mercuric sulphate-filtrate by the Hulme and Narain method (1931); the appearance or the increase of the reducing power originated in the liberation of ribose. Mercuric acetate was also available for the precipitation of guanine derivatives. One and half cc. of 30% mercuric acetate solution was sufficient to precipitate sodium guanylate completely from 10 cc. of 1% solution. Contrary to the case of mercuric sulphate, the use of excess of this reagent did not invite the risk of incomplete precipitation of the guanylate.

The enzyme solution was prepared in the following way. Hashed rabbit muscle was extracted with an equal weight of water for an hour at 37° C., and the extract was submitted to fractional precipitation with acetone. The fraction which was precipitated at the acetone concentration between 40 and 50% was found to be most active. We may recall here the finding of Euler and Brunius (1927) that the most active fraction of nucleosidase was precipitated from the autolysate of cattle kidney at the same concentration of acetone. The autolysis of the extracts prepared from rabbit muscle caused the loss of activity of *N*-ribosidase. The dried powder of the fraction was kept intact for a long while. The watery extracts of the powder, which were prepared by extracting 4–20 gm. of the powder with 100 cc. of water for an hour at 37°C., were used as the enzyme solutions.

I. *Reaction Velocity of N-Ribosidase.*

The monomolecular reaction velocity constant of the enzyme as calculated by means of the equation $K = 1/t \ln a/a\text{-}x$ is listed in Table I together with the experimental conditions. The constancy of K is clearly demonstrated in the table. The hydrogen-ion concentration of the mixture was adjusted at the beginning to pH 7.4 which was found to be the optimum value for the enzyme. Buffer solutions were not used for this purpose. The pH value remained unchanged during the reaction. In all the experiments reported in this communication the results obtained in the main experiments were always checked by controls which were set up without the addition of the substrates.

TABLE I.

Monomolecular Reaction Velocity Constant of *N*-ribosidase.
10 cc. of 20 % enzyme extract + 10 cc. of 1 % sodium guanylate solution.
1 cc. of toluene. pH 7.4. 37°C.

t (hour)	Ribosephosphoric ester formed (mg.)	Hydrolysis (%)	$K \times 10^2$ $\left(K=\frac{1}{t} ln \frac{a}{a-x}\right)$
1	2.38	4.2	4.31
2	5.04	8.9	4.66
3	7.12	12.6	4.57
4	8.92	15.8	4.32
5	11.28	20.0	4.47

II. Inhibition of the Enzyme Action by the Hydrolysis-Products.

The action of *N*-ribosidase was markedly inhibited by the hydrolysis-products —— guanine and ribosephosphoric ester Guanine exerted also inhibitory effect on nucleosidase. The effect of guanine on the enzymes was examined by comparing the degree of hydrolysis of the substrates in (a) the presence and (b) the absence of guanine. Guanine was used in a state of 0.1% suspension which was prepared according to Schmidt's method (1932). The guanosine solution was made by dissolving the compound in warm water at a desired concentration, the solution was cooled to 37° C. and added to the enzyme extract at 37° C. The conditions and the results of the experiments are revealed in Table II.

The results of the experiments which were designed to show the inhibitory effect of ribose-3- and -5-phosphoric acids on *N*-ribosidase are shown in Table III. Ribose-3-phosphoric ester was prepared from yeast xanthylic acid and ribose-5-phosphoric

TABLE II.

Inhibitory Effect of Guanine on *N*-ribosidase and Ribonucleosidase.

I: 10cc. of 8% enzyme extract+10cc. of 0.5% sodium guanylate or of 0.25% guanosine solution+25cc. of 0.1% guanine suspension.
1cc. of toluene. pH 7.4. 37°C. 20 hours.

II: 25cc. of water instead of guanine suspension.

Guanylate and guanosine were hydrolyzed with different enzyme samples.

Experiment	Previous addition of guanine	*N*-ribosidase		Nucleosidas	
		Production of ester (mg.)	Hydrolysis of guanylate (%)	Production of ribose (mg.)	Hydrolysis of guanosine (%)
I	+	4.38	15.5	3.84	29.0
II	−	7.94	28.1	5.05	38.1

TABLE III.

Inhibitory Effect of Ribosephosphoric Esters on *N*-ribosidase.

I: 10cc. of 8% enzyme extract+10cc. of 0.5% sodium guanylate solution+20cc. of ester solutions (15.58mg. of ribose-3-phosphoric ester or 16.48mg. of ribose-5-phosphoric ester).
1 cc. of toluene. pH 7.4. 37°C. 20 hours.

II: 20cc. of water instead of the ester solutions.

Ester	Experiment	Previous addition of ester	Production of ester (mg.)	Hydrolysis (%)
Ribose-3-phosphoric ester	I	+	4.51	15.9
	II	−	8.33	29.5
Ribose-5-phosphoric ester	I	+	3.74	13.2
	II	−	7.61	26.9

ester from muscle inosinic acid by mild acid hydrolysis.

When guanylate and guanosine were attacked simultaneously by the enzyme, the hydrolysis of each substrate took place to a less extent than in the case where the substrate was hydrolyzed separately (Table IV).

TABLE IV.

Simultaneous Hydrolysis of Guanylate and Guanosine.
10 cc. of 8% enzyme extract + 10 cc. of substrate solutions (I: 100 mg. of sodium guanylate, II: 50 mg. of guanosine. III: I+II).
1 cc. of toluene. pH 7.4. 37°C., 20 hours.

Experiment	Substrate	N-ribosidate		Nucleosidase	
		Production of ester (mg.)	Hydrolysis of guanylate (%)	Production of ribose (mg.)	Hydrolysis of guanosine (%)
I	Guanylate	13.98	24.7	—	—
II	Guanosine	—	—	6.41	24.2
III	Guanylate + guanosine	8.88	15.7	4.32	16.3

III. Purification of N-Ribosidase.

1) *Isoelectric precipitation of the enzyme.*

When the hydrogen-ion concentration of the enzyme solution was raised to pH 5 by the careful addition of *N* acetic acid solution, the solution became slightly turbid. Restoring the pH back to 7 with N NaOH solution, there occured a heavy precipitation of protein from the solution. The enzyme was distributed between the supernatant fluid and the precipitate at ratios varying from 1:1 to 1:4. The re-extraction of the precipitate with water at varying hydrogen-ion concentrations gave little promise.

2) *Adsorption of the enzyme by alumina.*

Klein (1935) found that desoxyribonucleosidase was adsorbed most effectively by aluminium hydroxide C_γ among the alumina preparations, and that arsenate was the specific eluting reagent, phosphate being also available but with less effectiveness. The hydrogen-ion concentrations of the media did not exert marked influence on adsorption and elution, but the optimum pH was 5 for adsorption, and 6–7 for elution. It is revealed in Table V that *N*-ribosidase, and ribonucleosidase too, assumed quite a similar attitude toward the alumina preparations; the adsorption of both enzymes was carried out most markedly by C_γ and at pH 5.0. The adsorbing capacities of the alumina preparations were examined by determining the enzyme activities of the Na_2HPO_4-extracts of the adsorbents carrying the enzymes by adsorption. The conditions of the determination of the enzyme activities are described in the table. The adsorption and the subsequent elution of the enzymes were carried out in the following way. Ten cc. of 4% extract of acetone-dried power were shaken with 100 mg. (10 cc.) of aluminium hydroxide, A, B or C_γ for 10 minutes, centrifuged, and the precipitate was eluted with 10 cc. of 1/20 M disodium hydrogen phosphate for 10 minutes at room temperature. The sample of the enzyme used in the case of nucleotide was of a different source from that used in the case of nucleoside.

Table V.

Adsorption-Elution of *N*-ribosidase and Ribonucleosidase by Aluminium Hydroxide.

10 cc. of purified enzyme solution + 10 cc. of 0.5% sodium guanylate solution or 0.25% guanosine solution.
1 cc. of toluene. pH 7.4. 37°C. 20 hours.

Adsorption		Activity of purified enzymes			
		N-ribosidase		Ribonucleosidase	
Adsorbent	pH	Production of ester (mg.)	Hydrolysis of guanylate (%)	Production of ribose (mg.)	Hydrolysis of guanosine (%)
A	4.0	1.04	3.7	—	—
	4.5	1.19	4.2	1.17	8.8
	5.0	1.75	6.2	1.37	10.3
	5.5	1.28	4.5	1.21	9.1
B	4.5	0.45	1.6	0.12	0.9
	5.0	0.74	2.6	0.20	1.5
	5.5	0.59	2.1	0.08	0·6
C_γ	4.5	1.78	6.3	1.91	14.4
	5.0	2.67	9.5	2.07	15.6
	5.5	2.23	7.9	1.68	12.7
Original extract		3.86	13.7	2.45	18.5

The minimum amount of C_γ which was practically sufficient for the complete adsorption of the enzyme from 10 cc. of the 4% extract of the acetone-dried powder, was 100 mg. The optimum concentration of disodium hydrogen phosphate in the eluting fluid was found to be 1/20 M. One tenth molar solution was slightly less active, and the eluting effect was reduced to 2/3 in 1/50 M solution. Disodium hydrogen arsenate was also effective, its optimum concentration being 1/20 M (Table VI.) The effect of arsenate was decidedly greater than that of phosphate in the case of nucleosidase.

TABLE VI.

Elution of the Enzymes from Aluminium Hydroxide Cγ with Phosphate and Arsenate Solutions of Varying Concentrations.

Adsorption: 10 cc. of 10% extract of muscle powder + 100 mg. of Cγ, shaken for 10 minutes.
Elution: Cγ + 10 cc. of Na_2HPO_4- or Na_2HAsSO_4-solutions, shaken for 10 minutes.
Experiment: 10 cc. of eluate+100 mg. of guanylate or 50 mg. of guanosine in 10 cc.
1 cc. of toluene, pH 7.0. 37°C. 20 hours.
Control: 10 cc. of original solution. pH 74.

Eluting fluid		N-ribosidase		Nucleosidase	
Kind	Concentration (M)	Production of ester (mg.)	Hydrolysis of guanylate (%)	Production of ribose (mg.)	Hydrolysis of guanosine (%)
Phosphate	1/10	7.42	13.1	—	—
	1/20	8.61	15.2	12.14	45.8
	1/50	6.98	12.3	—	—
Arsenate	1/10	8.76	15.5	—	—
	1/20	9.79	17.3	23.20	87.6
	1/50	7.72	13.7	—	—
Original enzyme solution		9.20	16.3	16.00	60.4

Klein observed the shift of the optimum hydrogen-ion concentration of the desoxyribonucleosidase from pH 7.5 to 6.5 after a single adsorption-elution. During the purification of N-ribosidase, quite a similar relation was observed between the purity and optimum pH of the enzyme. In this series of experiments the enzyme samples, which were of different sources in each case, were: 1) 8% extract of acetone-dried powder (original enzyme solution), 2) the once purified enzyme solution which was prepared by submitting the original solution to a single adsorption-elution, 3) the thrice successively purified enzyme solution. The adsorption and the elution of the enzyme were carried out in the same way as mentioned before. The successive purification was prformed by repeating this adsorption-elution. Since the first eluate contained sufficient quantity of phosphate to prevent the second adsorption of the enzyme, the fluid was freed from phosphate before the second addition of the adsorbent. For this purpose the fluid was dialyzed through fish skin for more than 10 hours, or it was added with magnesia mixture at pH 8.6 and 0° C., then dialyzed for several hours. Dialysis for 15 hours exerted practically no noticeable reducing influence on the enzyme activity. With the extension of the duration of dialysis, the enzyme activity tended to decrease; 30 hours' dialysis reduced the activity to 2/3 of the original value. From the second elution on, the content of phosphate in the eluates was reduced so markedly that it did not inhibit the subsequent adsorption. The activity of the original and purified enzyme samples was examined under the conditions described in Table VII. The hydrogen-ion concentration of the solutions to be tested covered a range between pH 6.2 and 7.8. The pH was adjusted to the desired value with1 M acetic acid and sodium hydroxide solutions, without using buffer solutions, in those experiments carried out with the original and once purified enzyme samples. The activity of the thrice purified enzyme was examined at more accurate hydrogen-ion concentrations; they were arranged with phosphate buffer mixtures of Sörensen. The change of the pH-activity relation with the purity of the enzymes is shown in the table. The optimum pH of the crude original solutions of *N*-ribosidase and ribonucleosidase was round 7.5, but it was pushed to 7 after a single purification and to round 6.5 after three successive treatments.

The purification of *N*-ribosidase by the adsorption-elution eliminated not only impurities but also the enzyme itself from

Table VII.

Purification of *N*-ribosidase and Ribonucleosidase.
pH-Activity Relation.
10 cc. of enzyme solution +10 cc. of substrate solutions (100 mg. of sodium guanylate or 40 mg. of guanosine).
1 cc. of toluene, pH 6.2—7.8. 37°C. 20 hours.

Enzyme sample	pH	N-ribosidase		Ribonucleosidase	
		Production of ester (mg.)	Hydrolysis of guanylate (%)	Production of ribose (mg.)	Hydrolysis of guanosine (%)
Original solution	6.2	5.91	10.5	3.28	15.5
	6.6	6.52	11.5	3.60	17.0
	7.0	7.16	12.7	4.24	20.0
	7.4	9.84	17.4	5.67	26.8
	7.8	8.55	15.1	4.80	22.7
Once purified solution	6.2	5.73	10.1	2.60	12.3
	6.6	6.86	12.1	4.17	19.7
	7.0	10.48	18.5	5.40	25.5
	7.4	8.96	15.9	4.02	19.0
Thrice purified solution	6.2	3.53	6.3	1.17	5.5
	6.6	4.19	7.4	1.40	6.6
	7.0	3.27	5.8	0.86	4.1

the solution. However, the ease of the elimination was different in both cases, the impurities being removed more readily than the enzyme, and thus the purification was accomplished. It is demonstrated in Table VIII that the ratio between the activity of the enzyme and the nitrogen content shifted after every treatment in favour of the former. The third purification deprived the enzyme of one third of its activity, while the total amount of nitrogen was reduced to one hundredth. The subsequent treatment resulted in the rapid reduction of the enzyme activity. The thrice purified sample responded to the following colour-reactions of proteins: Millon's reaction (+); biuret, ninhydrin, lead acetate and Hopkins-Cole's reactions (−).

It was noticed during the purification of the enzyme solution

TABLE VIII.

Purification of N-ribosidase. Enzyme Activity and Nitrogen Content.

Enzyme sample	pH	N-ribosidase		Nucleosidase		N-content in enzyme solution (mg.%)
		Production of ester (mg.)	Hydrolysis of guanylate (%)	Production of ribose (mg)	Hydrolysis of guanosine (%)	
Original sample	7.4	15.94	28.2	11.68	55.1	847.2
Once purified	7.0	15.79	27.9	—	—	282.4
Twice purified	6.8	12.79	22.6	10.16	49.9	25.4
Thrice purified	6.6	10.15	18.0	—	—	8.8
Four times purified	6.6	2.29	4.1	2.12	10.0	2.6

that nucleosidase was also purified by the treatment and thus the increase of its activity ran closely parallel with that of N-ribosidase. The enzyme sample used through these experiments was the 10% extract of the acetone-dried muscle powder. The enzyme solution was made in a large quantity and aliquoted after each purification, so that the main and blank experiments might be made. The purification of the enzyme and the examination of its activity were carried out under conditions similar to those of the experiments listed in Table VII, with the exception that the enzyme activity was tested at optimum hydrogen-ion concentrations varying with the purity of the enzyme.

Thus as far as the purification by adsorption-elution and the pH-activity relation are concerned, one cannot distinguish N-ribosidase from nucleosidase. Considering that N-ribosidase and nucleosidase disrupt the same glycosidic linkages of the substrates, it is no wonder that there is a close parallelism between them. The only difference which the present writer ever noticed was that inorganic phosphate or arsenate was indispensable for the action of nucleosidase while N-ribosidase was practically indifferent to the presence or absence of the salts. The 10% enzyme extract was dialyzed through fish skin for 20 hours against tap water, and 10 cc. of the dialysate were incubated for 20 hours with 100 mg. of guanylate or 40 mg. of guanosine in the usual way. The hydrolysis of the substrates in presence or absence of 1/20 M disodium hydrogen phosphate was compared with the case where the enzyme solution was not dialyzed but left in a flask during the dialysis at the temperature of water. The results are presented in Table IX. The optimum concentration of disodium hydrogen phosphate for activation of nucleosidase was found to be 1/20 M. That arsenate activated nucleosidase more markedly than phosphate while *N*-ribosidase was almost indifferent to the kind of the salts, may be cited, in this connection, as another difference between these two enzymes.

TABLE IX.

Influence of Phosphate on Activity of *N*-ribosidase and Nucleosidase.

Enzyme	N-ribosidase		Nucleosidase	
	Production of ester (mg.)	Hydrolysis of guanylate (%)	Production of ribose (mg.)	Hydrolysis of guanosine (%)
Not dialyzed	9.83	17.4	4.03	19.0
Dialyzed	8.93	15.8	0.13	0.6
Dialyzed + Phosphate	9.15	16.2	4.26	20.1

SUMMARY.

1. The monomolecular reaction velocity constant of nucleotide-*N*-ribosidase was calculated with the use of sodium guanylate as the substrate; $K \times 10^2 = 4.48$ on an average.

2. The enzyme action was markedly inhibited by the hydrolysis products, that is, guanine and ribosephosphoric ester.

3. *N*-ribosidase was highly purified by repeating the adsorption-elution of the enzyme with aluminium hydroxide C_γ and with

disodium hydrogen phosphate. The optimum hydrogen-ion concentration of the enzyme activity was shifted from pH 7.5 to 6.5 by the repetition of the purifying treatment. The best conditions for the adsorption-elution were sought. During the process of purification the activity of nucleosidase increased parallel with that of *N*-ribosidase. These two enzymes displayed a very close similarity in many respects at the time of their purification with adsorption-elution. The only difference so far noticed between the enzymes was the indispensability of inorganic phosphate or arsenate for the activity of nucleosidase while that of *N*-ribosidase was not affected by these salts.

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THE MICRO DETERMINATION OF THE IODINE NUMBER OF STEROLS IN THE TISSUES WITH SPECIAL REFERENCE TO ERGOSTEROL.

BY

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In an earlier work (1931–32) the author has reported that the method of Rosenmund and Kuhnhenn (1923) for the determination of the iodine number of lipids in which lipids were halogenized with pyridine sulfate dibromide was preferable to the widely used Hanus method, and that the former was likely applicable to cholesterol and its esters with satisfactory results. It seemed then probable that the iodine number of other unsaturated sterols might be determined by the same principle. When, however, ergosterol was selected as a substrate, the results were extremely high showing that none of the methods hitherto available was good for ergosterol.

In search of the literatures regarding the determination of the iodine number of unsaturated sterols, Smedley-MacLean (1928) has stated that when Wijs reagent was used, the iodine values of highly unsaturated sterols were nearly twice as great as those given with Huebl reagent, and even with the latter, the values were surprisingly high. The iodine values obtained by the Rosenmund-Kuhnhenn reagent for a sample of ergosterol of the melting point 158.5° and for zymosterol of the melting point 99–101° were 296.0 and 299.8 respectively, and the values were greatly influenced by the amount of the reagent, temperature and the length of time during which sterols were left in contact with the reagent. Accordingly, it was concluded that the method of Rosenmund-Kuhnhenn was no more satisfactory than that of Huebl or Wijs for the determination of the iodine number of these sterols.

Reindel and Weickmann (1929) have estimated the grade of desaturation of zymosterol and ergosterol by treating with benzo peracid. They added chloroform solution of 0.2–0.3 normal peracid to about 0.15 gm. of sterols dissolved in chloroform, left for more than 100 hours at 0° or 15°, and the remaining peracid was titrated with 0.1 normal sodium thiosulfate after the addition of potassium iodide. The number of double bond of respective sterols was calculated from the amount of peracid utilized, that is from the number of oxygen atoms taken by one molecule of sterols. When the reaction mixture was left for 10 days at 15°, the calculated number of double bond of zymosterol was 1.97–2.09 and that of ergosterol was 3.06–3.16 under the same condition. These values agreed fairly with the theory.

In animal tissues at least three different sterols, cholesterol, dihydrocholesterol and ergosterol, are reported to be present, and in feces the existence of coprosterol together with cholesterol and dihydrocholesterol is generally known. On the other hand numerous sterols of different grades of desaturation such as ergosterol, sitosterol, stigmasterol, zymosterol and so forth are isolated from vegetable tissues. When these sterols are present in different ratios, the determination of the iodine number would probably serve as the most convenient tool in finding out the distribution of saturated and unsaturated sterols.

In recent publication (1936), the author has shown that cholesterol in the tissues was isolated as digitonide from which digitonin was removed with simple procedure leaving only cholelsterol for further treatment. Other sterols which precipitate with digitonin may be isolated in the same way. The determination of the iodine number of sterols in animal tissues would then become easy, had any method suitable for both of cholesterol and ergosterol been devised. The ingenious method of Reindel and Weickmann has made it possible to give the number of double bond of ergosterol, but it did not seem to be intended for the study of mixed sterols available from relatively small amount of the tissues, and the others were entirely inadequate for ergosterol as stated above.

The author has, therefore, first worked out on ergosterol before entering the determination of the iodine number of tissue sterols with which the present communication is chiefly concerned.

DETERMINATION OF THE IODINE NUMBER OF ERGOSTEROL.

Application of Hanus and Rosenmund-Kuhnhenn Methods to Ergosterol—Having three double bonds the theoretical iodine number of ergosterol $C_{28}H_{44}O$ should be 192.

To a sample of ergosterol (Fraenkel Ergosterin) both Hanus and Rosenmund-Kuhnhenn methods were applied: 10 mg. of ergosterol dissolved in 10 cc. of chloroform were added to 20 cc. of Hanus and 10 cc. of Rosenmund-Kuhnhenn reagents, and the amount of halogen absorbed was determined by titrating with 0.1n $Na_2S_2O_3$ after the addition of 5 cc. of 10 per cent potassium iodide. The reagents were prepared as follows:

Hanus—26 gm. of iodine and 5 cc. of bromine, each dissolved in glacial acetic acid, were mixed and made up to 2000 cc. with glacial acetic acid.

Rosenmund-Kuhnhenn—16.5 cc. (16 gm.) of pyridine and 10.9 cc. (20 gm.) of concentrated H_2SO_4 were measured into separate flasks containing about 40 cc. of glacial acetic acid upon cooling, and then combined. 5 cc. (16 gm.) of bromine dissolved in 40 cc. of glacial acetic acid was added and made up to 2000 cc. with the same acetic acid as described in previous publication (1931–32).

The iodine number was calculated after the formula

$$\frac{a-b}{c} \times 1.27 = \text{I. N.}$$

in which a represented the number of cc. of the blank titration; b, the number of cc. of 0.1n $Na_2S_2O_3$ needed for the titration; and c, the number of gm. of sterol.

The results of above experiments are presented in Table I which shows that both methods gave greatly high values, and that between Hanus and Rosenmund-Kuhnhenn methods there was no particular difference in halogenizing ergosterol unlike in case of cholesterol (1931–32). The reaction took place at relatively high room temperature of about 30°C so that the same experiments were next carried on at low temperature: flasks containing the reaction mixture were placed in ice water for 1 to 10 minutes after that they were titrated with 0.1n $Na_2S_2O_3$ as before. The iodine number of ergosterol was 340 after 1 minute and 346 after 10 minutes of reaction. These values were also much higher than the expected ones, indicating that the absorption of halogen by ergosterol in the medium of chloroform was prompt and high even at the low temperature.

As the reagents so far used were not adequate for ergosterol, a new reagent was prepared by substituting bromine in Rosenmund-Kuhnhenn reagent with iodine, namely 25 gm. of iodine were used instead of 16 gm. of bromine. With this reagent (Yasuda reagent in Table I and II) the iodine number of ergosterol was very near to the theoretical, after 45 to 60 minutes of the time of reaction at room temperature.

TABLE I.

Determination of the iodine number of ergosterol by the reagents of Hanus, Rosenmund-Kuhenn and Yasuda.

Aliquot: 10 mg. of ergosterol (Fraenkel Ergosterin) in 10 cc. of chloroform.

Reagent: 10 cc. in case of Hanus, and 20 cc. in other cases.

Time of reaction	Iodine number				
	Hanus	Rosenmund	Yasuda		
min. 5	421	428	131		
15	446	440	170	173	
30	448	447			
45			182	182	
60	448	453	185	188	188
120	467	468	186	190	188
180			189		191

However, when the same reagent was used for oleic acid (iodine number=90), the results were very low no matter how the reagent was increased and the time of reaction was extended. Besides, the reagent has shown that it did not react toward cholesterol (iodine number=66) at all as indicated in Table II, consequently the satisfactory results obtained on ergosterol were considered to be accidental although they were constant at repeated experiments.

TABLE II.

Determination of the iodine number of cholesterol by the reagents of Hanus, Rosenmund-Kuhnhenn and Yasuda.

Aliquot: 25 mg. of cholesterol (Merck) in 5 cc. of chloroform.

Reagent: 10 cc. each.

Time of reaction	Hanus		Rosenmund		Yasuda	
	Titration*	I. N.	Titration	I. N.	Titration	I. N.
min.	cc.		cc.		cc.	
5	17.30	85.5	7.31	62.4	9.10	undetermined
15	17.13	94.1	7.22	66.8		
30	16.88	106.5	7.18	69.0	9.08	
60	16.60	120.5	7.18	69.0	9.08	
120	16.15	143.5	7.15	70.5	9.08	
blanc	19.00		8.55		9.10	

* with 0.1n $Na_2S_2O_3$ (factor=0.990)

Preference of Methyl Alcohol as a Solvent of Ergosterol—Since the search for a new reagent applicable to ergosterol as well as to other lipids did not seem promising, the medium of halogenizing was changed. The experiments were done by micro method to save the material, as the author has confirmed in previous publication (1931–32), that the micro determination of the iodine number of fatty acids and cholesterol with pyridine sulfate dibromide gave exactly same values to those by macro method.

Aliquots 2 mg. of ergosterol were dissolved in chloroform, carbon tetrachloride, acetic acid and methyl alcohol, then added to 2 cc. of diluted pyridine sulfate dibromide reagent, prepared by mixing equal volumes of the original reagent and glacial acetic acid. After different times of reaction 0.5 cc. of 5 per cent potassium iodide were added, then titrated with 0.02 n $Na_2S_2O_3$. Detailed procedures of the micro determination will be described later.

The inadequacy of chloroform as a solvent of ergosterol was already pointed out which was again demonstrated in above experiment, and the same was observed on acetic acid. In carbon tetrachloride the absorption of halogen in the first 5 to 10 minutes was near the theory, but it increased with the time. Only in methyl alcohol the amount of halogen taken by ergosterol agreed with the theoretical, and it was not changed by the length of the time of reaction. The trouble in the determination of the iodine number of ergosterol was thus removed by simply making use of methyl alcohol as the medium of halogenizing.

Methyl alcohol, however, is not good solvent of ergosterol so that a small portion of acetic acid was added whereby ergosterol has become quite soluble, and furthermore the mixture had the advantage that it dissolves digitonin precipitate of sterols as will be commented in the next chapter. Since ergosterol absorbs halogen much larger than the theory in acetic acid, the amount of the latter to be used with methyl alcohol was studied and it was found out that 4 cc. of methyl alcohol to 1 cc. of acetic acid were the satisfactory ratio for the present purpose. The above results are given in Table III and Fig. I.

Experiments were repeated on different aliquots of ergosterol to confirm the above findings followed by the experiments on cholesterol, since it was necessary to see whether or not the conditions under which ergosterol was halogenized with satisfactory results were suitable for cholesterol. Table IV would show the results of these experiments in which it may be obvious that the iodine number of cholesterol is as well determined in methyl alcohol as in chloroform, while that of ergosterol is determined only in methyl alcohol. When, therefore, methyl alcohol is used as a solvent and pyridine sulfate dibromide as a halogenizing agent, the iodine numbers of two important sterols in animal tissues would be determined without difficulties. It was also

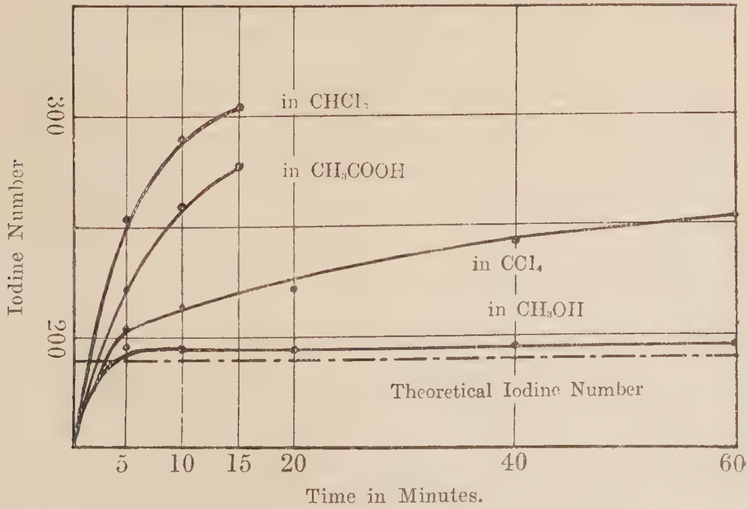
TABLE III.

Halogen absorption of ergosterol in different solvents at different times of reaction.

2 mg. i. e. 2 cc. of 0.1 per cent methyl alcoholic solution of ergosterol were used as aliquots. After methyl alcohol was evaporated, ergosterol was redissolved in different solvents and halogenized with 2 cc. of diluted Rosenmund-Kuhnhenn reagent.

Solvent	Time of reaction	Absorbed halogen in terms of 0.02n $Na_2S_2O_3$	Iodine number	Diff. from the theory
	min	cc		per cent
Methyl albohol 4 cc+1 cc acetic acid	5	1.56	198	3.6
	10	1.55	197	2.6
	20	1.55	197	2.6
	60	1.55	197	2.6
Carbon tetrachloride 5 cc	5	1.60	204	6.2
	10	1.68	214	11.5
	20	1.75	222	15.6
	40	1.87	238	23.9
	60	2.00	254	32.2
Chloroform 2 cc+1 cc acetic acid	5	2.00	254	32.2
	10	2.28	290	51.0
	15	2.38	302	57.3
Acetic acid 3 cc	10	2.05	260	35.4
	15	2.20	280	45.7

Fig. 1. Halogen absorption of ergosterol in different solvents.



striking that the halogenization of both sterols was completed within a short time, five or ten minutes, at room temperature and remained unaltered for at least one hour. The iodine number of ergosterol given in Table III and IV differed about 1 to 2 per cent from each other, but the difference was within experimental error.

Table IV.

Halogen absorption of ergosterol and cholesterol in different solvents.

Aliquot	Solvent	Time of reaction	Titration with 0.02n $Na_2S_2O_3$	Absorbed halogen in terms of 0.02n $Na_2S_2O_3$	Iodine number	Diff. from the theory
mg.		min.	cc.	cc.		per cent
Ergosterol 2.0	Methyl alc. 4 cc.+1 cc. acetic acid	5	2.40	1.58	201	4.6
3.0		,,	1.60	2.38	201	,,
4.0		,,	0.83	3.15	201	,,
blanc		,,	3.98			
Ergosterol 2.0	Methyl alc. 4 cc.+1 cc. acetic acid	10	2.35	1.58	201	4.6
3.0		,,	1.55	2.38	201	,,
4.0		,,	0.75	3.18	202	5.2
blanc		,,	3.93			
Cholesterol 5.0	Methyl alc. 4 cc.+1 cc. acetic acid	5	2.56	1.36	69	4.5
5.0		10	2.55	1.31	67	1.5
blanc		5	3.92			
,,		10	3.86			
Cholesterol 5.0	Chloroform 2 cc.	10	2.60	1.30	66	0
Ergosterol 2.0		,,	1.40	2.50	318	65.6
blanc		,,	3.90			

Sterols were halogenized with 2 cc. of diluted Rosenmund-Kuhnhenn reagent.

Determination of the Iodine Number of Tissue Sterols.

Halogen Absorption of Digitonin—Since sterols in the tissues are isolated with the aid of digitonin, the absorption of halogen by digitonin was investigated.

10 to 30 mg. of digitonin (Merck) dissolved in alcohol were

taken into small glass stopped Erlenmeyer flasks. Alcohol was evaporated, the residue was redissolved in 5 cc. of glacial acetic acid and added to 2 or 3 cc. of diluted pyridine sulfate dibromide reagent. After 10 to 30 minutes the flasks were titrated with 0.02n $Na_2S_2O_3$. In Table V the amount of halogen absorbed by digitonin is expressed in terms of 0.02n $Na_2S_2O_3$.

TABLE V.

Halogen absorption of digitonin.

Digitonin	Reagent used	Time of reaction	Titration with 0.02n $Na_2S_2O_3$	Absorbed halogen per 10 mg. digitonin in terms of 0.02n $Na_2S_2O_3$
mg.	cc.	min.	cc.	cc.
10	2.0	10	4.15	0.050
20	,,	,,	4.08	0.060
blanc	,,	,,	4.20	
10	2.0	15	4.11	0.090
20	,,	,,	4.05	0.075
blanc	,,	,,	4.20	
10	2.0	30	4.12	0.085
20	,,	,,	4.01	0.095
blanc	,,	,,	4.20	
10	3.0*	15	6.12	0.030
20	,,	,,	6.00	0.075
30	,,	,,	5.90	0·083
blanc	,,	,,	6.15	
10	3.0*	30	6.08	0.070
20	,,	,,	5.95	0.100
30	,,	,,	5.87	0.093
blanc	,,	,,	6.15	

* Newly prepared reagent.

The results in Table V indicated that digitonin absorbed slight but constant amount of halogen when treated with pyridine sulfate dibromide. The average amount of halogen taken per 10 mg. of digitonin was corresponding to 0.08 cc. (or per 1 mg. of digitonin 0.008 cc.) of 0.02n $Na_2S_2O_3$. Whether the absorption of halogen was caused by the impurities contained in digitonin or by the substitution in digitonin molecule was not decided.

Digitonides as Substrates—The above data would suggest that the iodine number of sterols may be determined by halogenizing digitonides, whereby the amount of halogen taken by digitonin is subtracted from that by digitonides.

Aliquots methyl alcoholic solution of cholesterol digitonide were pipetted into glass stoppered Erlenmeyer flasks of 120 cc. volume, the solvent was removed by evaporation, and the remaining digitonide was redissolved in 1 cc. of acetic acid and 4 cc. of methyl alcohol, or in 5cc. of acetic acid with gentle heating on the steam bath. After cooling 2 cc. of the halogenizing reagent were added. The amount of cholesterol digitonide contained in the aliquots was determined by oxidative method (Yasuda, 1931).

Since 1 mg. of cholesterol combines with 3.12 mg. of digitonin and 10 mg. of digitonin take halogen equivalent to 0.08 cc. of 0.02n $Na_2S_2O_3$, the amount of halogen taken by cholesterol was easily calculated. As shown in Table VI, the iodine number of cholesterol obtained by halogenizing its digitonide were between 67 and 74 with one exception. These values agreed with the ones obtained by halogenizing cholesterol as observed in Table IV, and with the theoretical.

Ergosterol was then used as a material for the same experiment, and this time the iodine number was determined either on ergosterol digitonide or ergosterol freed from digitonide, in either case the substrates were dissolved in acetic acid and methyl alcohol mixture before adding to the halogenizing reagent. As the solvent of digitonides a small amount of acetic acid was indispensable, since digitonides were not kept in solution in cold methyl alcohol alone. In Table VI it may be seen that the iodine number of ergosterol determined on its digitonide indirectly, or on ergosterol directly after it was freed from digitonide was practically the same.

From the results in Table V and VI the following conclusions were drawn:

1. Sterols precipitated with digitonin are freed from the latter, and the determinations of the iodine number are made on sterols thus treated.

2. The determinations are also available on digitonides, since the amount of halogen to be taken by digitonin is given by calculation.

TABLE VI.

Iodine number of cholesterol and ergosterol determined on digitonides or sterols freed from digitonides.

* Average of 5 determinations by oxidative method. The amount of digitonin combined with 1.8 mg. of cholesterol is 5.6 mg. which take halogen corresponding to $0.008 \times 5.6 = 0.045$ cc of 0.02 n $Na_2S_2O_3$.

** Digitonide was formed by adding digitonin to 2 mg. of ergosterol dissolved in methyl alcohol. 2 mg. of ergosterol combine with 6.2 mg. of digitonin which take halogen corresponding to 0.05 cc of 0.02n $Na_2S_2O_3$.

Materials (sterol in digitonide)	Time of reaction	Amount of halogen absorbed by (in terms of 0.02n $Na_2S_2O_3$)		Iodine number
		Digitonide	Sterol	
mg.	min.	cc	cc	
Chole. digitonide*..7.45 (1.8)	10	0.52	0.475	67
..	,,	0.55	0.505	71
..	,,	0.54	0.495	70
..	15	0.52	0.475	67
..	,,	0.55	0.505	71
..	,,	0.57	0.525	74
,,	30	0.57	0.525	74
,,	,,	0.57	0.525	74
..	45	0.60	0.555	78
..	,,	0.55	0.505	71
Ergo. digitonide**..8.12 (2.0)	10	1.57	1.52	193
,,	,,	1.60	1.55	197
Ergosterol freed from digitonide 2.0	10		1.50	191
..	,,		1.43	182
..	,,		1.55	197
,,	,,		1.50	191

PROCEDURES.

Isolation of Sterols from the Tissues—Sterols are isolated as digitonides from alcohol-ether or alcohol-acetone extracts of the tissues regarding which the author has reported in his recent publication on the cholesterol determination (1936). As to the removal of digitonin from digitonides the reader is also requested to refer to the same article. A part of digitonides or sterols freed from digitonin is halogenized, and the rest is oxidized with chromic acid or weighed to give the content of sterols.

Halogenizing—Following reagents are needed: diluted pyridine sulfate dibromide made by diluting the original reagent described elsewhere in this communication with equal volume of glacial acetic acid; 5 per cent potassium iodide solution; 1 per cent starch solution; 0.02n sodium thiosulfate adjusted against $0.02nKIO_3$.

About 3 mg. of sterols freed from digitonin or digitonides corresponding that amount of sterols are dissolved in 1 cc. of glacial acetic acid by gentle heating on the steam bath, cooled to room temperature and added to 4 cc. of methyl alcohol. 2 cc. of diluted pyridine sulfate dibromide reagent are then added and left standing. After 10 to 15 minutes, 0.5 cc. of 1 per cent KI solution are added and titrated with 0.02n $Na_2S_2O_3$.

Determination of Mixed Sterols—Sterols are determined either by oxidative or gravimetric method. The colorimetric determination is not applied since the color reaction to be used for mixed sterols is not introduced. The author recommends the oxidative method for the determination of mixed sterols which is carried on after the description on the oxidative micro determination of cholesterol (Yasuda, 1931).

When sterols are oxidized as digitonides with chromic acid and Nicloux reagent, 1 mg. of mixed sterols consumes 10.5 cc. of 0.1n $K_2Cr_2O_7$; and when sterols are oxidized after they were freed from digitonin, the average consumption should be 3.9 cc. of the same chromic acid per 1 mg. of mixed sterols as indicated below:

1 mg. of cholesterol needs....3.92 cc., and as digitonide....10.60 cc.
„ ergosterol „ ....3.88 „ „ ... 10.40 „

„ dihydrocholesterol or coprosterol ......3.96 „ „ ....10.60 „

therefore, 1 mg. of mixed sterols requires approximately 3.9 cc., and as digitonides 10.5 cc. of 0.1n $K_2Cr_2O_7$ for oxidation.

Calculations –The iodine number of sterols, that is the number of gramms of iodine to be absorbed per 100 gm. of sterols is calculated after the formulae

$$\frac{a-b}{c}\times\frac{1.27}{5}=\text{I.N.}\ldots.\ (1)$$ when sterols are halogenized after the removal of digitonin

$$\frac{(a-b)-0.008\times d}{c}\times\frac{1.27}{5}=\text{I.N.}\ldots.\ (2)$$ when sterols are halogenized as digitonides

in which a is the number of cc. for blanc titration; b, the number of cc. of 0.02n $Na_2S_2O_3$ needed for the titration after halogenizing; c, the amount of sterols halogenized and expressed in gm.; and d, in the formula (2), is the number of mg. of digitonin combined with sterols. $0.008\times d$ is the amount of halogen taken by digitonin and expressed in terms of 0.02n $Na_2S_2O_3$ (see Table V).

An average amount of digitonin to be taken by 1 mg. of mixed sterols is 3.1 mg., since 1 mg. of cholesterol, ergosterol and dihydrocholesterol or coprosterol add to 3.14, 3.06 and 3.09 mg. of digitonin respectively. Accordingly, d is given by multiplying 3.1 by the number of mg. of sterols i. e. $d=3.1\times c\times 1000$ (mg.), as c represents the amount of sterols and expressed in gm.

Experiments on some Tissues—The following experiments were undertaken as examples of the determination of the iodine number of tissue sterols by the procedures described above: as materials alcohol-ether extracts of human brain and rabbit liver were used which were made by extracting about 2.5 to 3 gm. of the medullar part, about 4 gm. of the cortical part of the human brain, and about 4 gm. of rabbit liver with 250 cc. of alcohol ether.

The iodine number was determined on free sterols contained in these extracts. About 30 to 100 cc. of the extracts were measured into flasks, added to 10 cc. of 1 per cent digitonin solution and evaporated to almost dryness on the steam bath. The precipitate of digitonides were treated with diluted acetone (mixture of equal volumes of acetone and water), heated for few minutes on the steam bath, and transfered into a glass filter. The precipitate was next washed with acetone and ether several times to remove impurities, after that it was dissolved in hot acetic acid and methyl alcohol and filtered into a flask in the filtering apparatus as in case of cholesterol determination (1936).

Methyl alcohol was removed by evaporation, then added to about 10 times as much ether as remaining acetic acid to precipitate digitonin. After an hour the precipitate of digitonin was removed by filtration. The filtrate which contained sterols was evaporated, redissolved in warm methyl alcohol, transfered into a 25 cc. volumetric flask and made to the volume with methyl alcohol after cooling. Aliquots methyl alcoholic solution of sterols thus prepared were pipetted into glass stoppered pyrex Erlenmeyer flasks of about 120 cc. volume. Some of them were used for halogenizing, and the others were subjected to oxidative determination of sterols. Since sterols were freed from digitonin, the iodine number was calculated after the formula (1). In Table VII the average values of each 2 or 3 determinations are presented.

Note 1. For the determination of the iodine number of total sterols, tissue extracts should be saponified with KOH, and then neutralized before digitonin is added. The procedures of saponification and subsequent formation of digitonides are the same to those of total cholesterol determination (1936).

2. When sterols are halogenized as digitonides, methyl alcohol and acetic acid solution of digitonides collected in a flask in the filtering apparatus is transfered into a volumetric flask of suitable size and made to the volume with methyl alcohol. An aliquot that contains digitonides corresponding to about 3 mg. of sterols is taken into a Erlenmeyer flask, the solvent is evaporated, then the substrate is redissoved in known amount of acetic acid and methyl alcohol and halogenized. About one third of the same aliquot which therefore contains about 1 mg. of sterols as digitonides is taken into another flask, the solvent is removed by evaporation with suction, then oxidized with chromic acid

and Nicloux reagent.

TABLE VII.

The iodine number of free sterols in some tissues.

Tissues	Sterols in aliq. tissue extract	Absorbed halogen** in terms of 0.02n $Na_2S_2O_3$	Iodine number
	mg.	cc.	
Human brain..M*–1	3.32	0.91	69.5
C– 1	3.12	0.90	73.4
M– 2	3.23	1.00	78.6
C– 6	2.23	0.67	76.4
M– 2	4.43	1.35	77.5
C– 6	2.55	0.67	66.7
Rabbit liver........	1.63	0.40	62.4

* M–1, 2, 6...Medullar part of the brain samples No. 1, 2 and 6
C–1, 2, 6....Cortical part „ „ „

** Sterols were halogenized in methyl alcohol (4 cc.) and acetic acid (1 cc.) for 10 minutes.

Judging from the iodine number given in Table VII, it is apparent that free sterols isolated from the brain and liver were probably cholesterol as might be expected.

COMMENTS.

It may be of significance that the behavior of ergosterol toward the absorption of halogen is characteristic to the nature of the solvents in which ergosterol is dissolved and halogenized. Different from cholesterol and fatty acids, the iodine number of ergosterol can not be determined in chloroform, but it can be done in methyl alcohol with the reagent of pyridine sulfate dibromide. With the same reagent and by practically same procedures as in case of other lipids (Yasuda, 1931–32) the iodine number of ergosterol is obtained within short time.

As lately reviewed by Bills (1935) ergosterol is widely distributed in animal tissues as well as in vegetable world. Although the content of ergosterol is only minute in normal animal tissues since cholesterol occupies most of the tissue sterols as observed in the experiments in Table VII, it might be increased in some tissues particularly under pathological or experimental conditions as, for instance, in case of feeding with ergosterol (Page, 1930). At any rate, the existence of ergosterol should be taken into consideration when the iodine number of tissue sterols is questioned, and the same may be stated regarding dihydrocholesterol and coprosterol. The present method is therefore based on the assumption that sterols in the tissues, blood, feces and so forth are mixed with those ones.

The procedures consisted of 1) isolation of sterols from the tissue extracts as digitonides 2) halogenization of digitonides and 3) determination of sterols by oxidizing digitonides. Sterols separated from digitonides are also subjected to halogenization and oxidative determination.

As to the procedures of 1) and 3) the author has commented in his previous communications (1931, 1936). The halogenization of digitonides or sterols with pyridine sulfate dibromide gives same duplicates so that the accuracy of this method depends on the determination of the sterols content for which oxidative method is recommended. The oxidative determination of lipids has not been free from objection that the oxidation of contaminated substances would affect the final results. However, the influence of impurities would be negligible as far as sterols that can be freed from contaminated impurities with the aid of digitonin are concerned. When enough materials are available, and the oxidative method is not prefered, then it should de substituted with the gravimetric method since the colorimetry can not be applied for the present purpose.

SUMMARY.

After computing the absorption of halogen by ergosterol and cholesterol under various conditions, a method for the micro determination of the iodine number of tissue sterols was finally devised.

The determination of the iodine number of tissue sterols seems to be important in relation to the studies of the nature

and the distribution of sterols of different grades of desaturation in animal tissues or in biological materials as a whole.

The present article is dedicated to Professor S. Kakiuchi whose Twenty Fifth Anniversary of Securing Professorship at Tokio Imperial University will be celebrated this spring.

The author wishes to add that he has enjoyed the helpful criticism of Professor Kakiuchi for this work with sincere thanks.

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MAGNESIUMSTOFFWECHSEL UND GALLENSÄURE.

Ausscheidung des Magnesium unter Einfluss von Cholsäure bei normalen sowie bei splanchnikotomierten Kaninchen.

Von

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Magnesium ist bekanntlich ein wichtiger Zellbestandteil und im Blut in einer derartig kleinen Menge enthalten, dass es für die Aufrechterhaltung des Säure-Basen-Gleichgewichtes gar nicht in Frage kommt.

Dieses Magnesium wird, wie ebenfalls bekannt, zum grösseren Teil durch den Darm und nur zum kleineren im Harn ausgeschieden und tritt mit dem Calcium und Phosphor im Haushalte des Körpers vielfach in Beziehung.

Nach Gerhardt u. Schlesinger (1899) scheint mit dem Mangel an Alkalien im Organismus die Vermehrung der Magnesia im Harn parallel zu gehen.

Nach Lescoeur u. Moquet (1923) besteht eine Beziehung zwischen der Magnesiumausscheidung im Urin und dem Urin-PH, derart, dass die Magnesiumausscheidung der Vermehrung der Wasserstoffionenkonzentration ungefähr parallel verläuft. Kuramoto (1934) hat schon beim Hunde bewiesen, dass der Harn durch Zufuhr von Cholsäure alkalisch wird, wobei das PH des Harns vermehrt wird.

Es sei hier daran erinnert, dass nach Zondek (1929) die nervösen Erregungen der vegetativen Organe eine Konzentrationsänderung der Elektrolyte hervorrufen und dass zwischen Calcium und Magnesium ein antagonistisches Verhältnis besteht, indem eine Vermehrung der Calciumionen im Blut zur Entgiftung dieser eine Vermehrung der Magnesiumionen bedingt, alles Umstände, welche besonders bei der Ausscheidung dieser Elemente eine gewisse Bedeutung haben.

So hat Sekitoo (1929) eine durch den Einfluss der Cholsäure bedingte Vermehrung von Calcium im Harn gefunden, aber ein wechselndes Verhalten in der Magnesiumausscheidung beobachtet. Danach hat Maruno (1931) eine Magnesiumvermehrung im Blut des Kaninchens beobachtet.

Nach den Ergebnissen obiger Autoren muss das Magnesium im Harn durch Cholsäure vermindert werden, während nach Lang (1932) der Blutmagnesiumspiegel wohl durch die Ernährung, nicht aber durch Hormone beeinflusst wird. Daher ist nach ihm der Magnesiumspiegel im Blut unabhängig von dem endokrinen System.

Neuerdings hat Iwado (1936) die Veränderung des Calcium und Magnesium in verschiedenen Organen bei Zufuhr von Cholsäure untersucht und festgestellt, dass diese Erdalkalien dadurch in verschiedenen Organen angesetzt werden, wobei der Ansatz des Calcium viel stärker auftritt als der des Magnesium. Nach Kuramoto (1934) soll die Magnesiumausscheidung im Darmsaft durch Cholsäure vermehrt, aber die Ausscheidung des Calcium vermindert werden und nach Okii (1934) die letztere mit der Funktion des vegetativen Nervensystems in inniger Beziehnung stehen.

Mit dem Versuch von Iwado (1934) wird eine merkwürdige Verschiebung des Ca/Mg-Quotienten durch Cholsäure gezeigt. Hier äussert sich ein relativer Mangel an Magnesium gegenüber dem Calcium. Die Mehrausscheidung des Magnesium durch die Darmwand kann auch als ein Faktor gegen diesen Magnesiummangel betrachtet werden. Hierbei müsste also entweder das Magnesium an den Geweben festgehalten werden, um den Ca/Mg-Quotienten möglichst in ein normales Verhältnis zu bringen, oder das Calcium von den Geweben rasch herausgezwungen werden.

Somit dürfte mit Wahrscheinlichkeit angenommen werden können, dass die Verschiebung des im Gewebe angesetzten Magnesium in die Blutbahn erst nach der Ausfuhr des überschüssigen Calcium stattfinden kann.

Somit muss die Ausscheidung des Magnesium bei Zufuhr von Cholsäure anfangs herabgesetzt, später dagegen vermehrt werden. Nach Kitayama (1927) soll die Kalkausscheidung im Harn durch Splanchnikotomie vermindert werden, wobei also die Magnesiumausscheidung im Harn im Sinne eines Antagonismus zwischen beiden Ionen vermehrt werden müsste und die Wirkung der Cholsäure auf die Magnesiumausscheidung bei splanchnikotomierten Kaninchen viel schwächer als bei normalen auftreten oder ganz ausbleiben dürfte, wie Sekitoo (1930) es beim Kalkgehalt des Blutes des splanchnikotomierten Kaninchens beobachtet hat.

Wenn das Verhalten der Magnesiumausscheidung im Harn bei Zufuhr von Cholsäure ausschliesslich durch die Gegeneinanderverschiebung der Erdalkalienionen bedingt sein sollte, die mit der Funktion der vegetativen Nerven eng verknüpft ist, so dürfte die Cholsäurewirkung auf die Magnesiumausscheidung im Harn des splanchnikotomierten Kaninchens keine andere sein als auf die Kalkausscheidung desselben Tieres.

Die Magnesiumausscheidung hängt bekanntlich von der Muskelarbeit ab. Bei normalem Verhältnis wird nach den Untersuchungen von Toda (1930) und Gellhorn (1932) die Muskelermüdung durch Calcium verzögert, was bei Gegenwart von adäquaten Mengen von Calcium auch durch Magnesium ersetzt werden kann.

Die Frage ob die Verschiebung der Magnesiumionen in Geweben und Organen durch Cholsäure auf der obengenannten Ursache beruht oder nicht, bleibt noch offen.

Die Bedeutung des Magnesium im Tierorganismus für den Stoffwechselvorgang stellt ein wichtiges Problem dar. Einerseits haben Mc. Collum u. seine Schüler (1932) berichtet, dass das Magnesium als Nahrungsbestandteil für das Wachstum und Leben des Tierorganismus unentbehrlich ist, andrerseits haben Meyerhof u. Lohmann (1932), Lohmann (1931), Erdmann (1927/28), Euler, Nilsson u. Auhagen (1931), Myrbäck u. Euler

(1931) auf die Rolle des Magnesium in der biologischen Kohlehydratspaltung aufmerksam gemacht. Nach diesen Autoren soll das Magnesium für die Synthese und die Spaltung der Zucker mit Phosphate und der Adenosintriphosphorsäure unentbehrlich sein, welch letztere bei den dem Abbauprozess des Zuckers im Muskel sowie in der Hefegährung mit Magnesium und anorganischem Phosphat als unbedingt notwendiger Bestandteil der bisher sogenannten Cozymase betrachtet wird, und nach Meyerhof u. Lohmann(1932) dient das abgespaltene Phosphat der Adenylpyrophosphorsäure zum Aufbau des Phosphagens und dessen Phosphorsäure geht erst bei der Phosyhorylierung des Zuckers über.

Nach der Untersnchung von Uraki(1931) fördert die Gallensäure die Phosphorylierung des Zuckers im Muskel und in der Leber, und nach Takata(1932) soll die Wirkung der Phosphatase der Leber und der Niere durch Gallensäure gehemmt werden, welch letztere bekanntlich im Kohlehydratstoffwechsel eine grosse Rolle spielt, indem sie die Glykogenie in der Leber und im Muskel fördert und die Glykolyse u. Glykogenolyse hemmt.

Weiter besteht nach der Untersuchung von Watanabe (1932/34) eine innige Beziehuug zwischen den Wirkungen von Gallensäure und von Adenylsäure bzw. Adenylpyrophosphorsäure beim Kohlehydratstoffwechsel im Muskel und in der Leber, indem die Adenylsäure die Glykogenie der Leber u. des Muskels fördert und die Adenylpyrophosphorsäure die Glykogenie in beiden vermindert, wobei nach ihm die Adenylsäure fermentativ in Adenylpyrophosphorsäure umgewandelt wird, um den Zuckerabbau zu fördern, was auch durch Gallensäure gefördert wird.

Die fermentative Spaltung dieser Nucleinsäure wird nach Karasawa (1926/27), Hatakeyama (1927) und Okamura (1930) bei Gegenwart von Gallensäure gesteigert.

Somit muss das am Kohlehydratstoffwechsel sich beteiligende Gozymasesystem durch Gallensäure beeinflusst werden, was eine veränderte Verteilung der Magnesiumionen im Gewebe, z. B. im Muskel, zur Folge haben muss. Infolgedessen dürfte die Ausscheidung des Magnesium im Harn durch Gallensäure beeinflusst werden.

Nach den oben angedeuteten zwei Richtungen hin habe ich zuerst die Magnesiumausscheidung im Harn des normalen sowie des splanchnikotomierten Kaninchens bei Zufuhr von Gallensäure untersucht.

EXPERIMENTELLER TEIL.

Zum Versuch wurden kräftige, gut erwachsene männliche Kaninchen verwendet, die 7–10 Tage lang mit einer bestimmten Menge Nahrung gefüttert worden waren. Die Nahrung bestand aus 50 g trockener Okara, 50 g Rübenblättern und 120 ccm Wasser.

Der Versuch wurde in 3 Perioden: Vor-, Versuchs- und Nachperiode geteilt. In der Versuchsperiode wurde den Kaninchen 3 ccm einer 1%igen Na-Cholatlösung pro Kilo subcutan verabreicht. In jeder Periode wurde der Harn 24 Stunden lang gesammelt und morgens früh durch Katheterisieren und Nachwaschen der Blase mit 2%iger Borsäurelösung abgegrenzt.

Der gesammelte Harn und das Waschwasser wurden mit Wasser auf 500 ccm aufgefüllt, und der Magnesiumgehalt nach der Methode von Mc Crudden (1901) bestimmt. Nach dem Katheterisieren des Harns wurde immer die obenangegebene Nahruug verabreicht und zwar in der Versuchsperiode erst nach dem Harnauslassen und der Cholatzufuhr.

Um den Einfluss der vegetativen Nervenfunktion auf die Magnesiumausscheidung im Harn festzustellen, wurde das Kaninchen nach Schulze (1900) splanchnikotomiert.

8–13 Tage nach der Operation wurde bei diesen splanchnikotomierten Kaninchen der gleiche Versuch ausgeführt, wie bei den normalen Kaninchen.

Die Resultate sind in den folgenden Tabellen zusammengefasst.

TABELLE I.

Körpergewicht 2080.

Datum (1932)	Harnmenge (ccm)	Reaktion	spec. Gew.	Mg-gehalt		Bemerkungen
				mg	mg%	
12/XI	131	alkalisch	1025	64,746	49,424	
13/XI	156	,,	1020	69,435	44,510	
14/XI	150	,,	1022	60,605	40,403	
15/XI	141	,,	1025	46,870	33,241	3 ccm 1% Na-cholatlös. pro Kilo
16/XI	184	,,	,,	58,315	32,218	
17/XI	121	,,	,,	36,735	30,360	
18/XI	173	,,	,,	59,515	34,402	
19/XI	147	,,	,,	78,700	53,537	
20/XI	100	,,	1030	42,300	42,300	

TABELLE II.

Körpergewicht 2219

Datum (1932)	Harnmenge (ccm)	Reaktion	spec. Gew.	Mg-gehalt		Bemerkungen
				mg	mg%	
12/XI	138	alkalisch	1025	67,255	48,736	
13/XI	153	,,	,,	62,350	40,752	
14/XI	171	,,	,,	66,710	39,012	
15/XI	186	,,	,,	55,810	30,005	3 ccm 1% Na-cholatlös. pro Kilo
16/XI	96	,,	1030	33,570	34,969	
17/XI	122	,,	,,	50,685	41,537	
18/XI	155	,,	1025	76,955	49,648	
19/XI	169	,,	,,	77,715	45,985	
20/XI	194	,,	1020	70,525	36,353	

Tabelle III.

Körpergewicht 2450

Datum (1932)	Harnmenge (ccm)	Reaktion	spec. Gew.	Mg-gehalt		Bemerkungen
				mg	mg%	
22/XI	135	alkalisch	1025	63,330	44,442	
23/XI	151	„	„	61,585	40,785	
24/XI	130	„	„	63,440	48,800	
25/XI	180	„	1020	47,956	26,642	3 ccm 1% Na-cholatlös. pro Kilo
26/XI	123	„	1030	41,640	33,854	
27/XI	115	„	„	73,030	63,504	
28/XI	182	„	„	63,110	51,737	
29/XI	165	„	„	84,645	51,300	
30/XI	147	„	„	79,570	54,129	

Tabelle IV.

Körpergewicht 2100

Datum (1932)	Harnmenge (ccm)	Reaktion	spec. Gew.	Mg-gehalt		Bemerkungen
				mg	mg%	
5/XII	173	alkalisc	1022	62,565	36,165	
6/XII	145	„	1030	63,545	43,824	
7/XII	155	„	„	65,835	42,474	
8/XII	121	„	„	42,745	35,326	3 ccm 1% Na-cholatlös. pro Kilo
9/XII	160	„	„	52,575	32,859	
10/XII	80	„	1035	53,845	67,306	
11/XII	162	„	„	57,660	35,950	
12/XII	148	„	„	82,295	55,605	
13/XII	164	„	„	88,615	54,034	

TABELLE V.

Körpergewicht 1390

Datum (1932)	Harnmenge (ccm)	Reaktion	spec. Gew.	Mg-gehalt		Bemerkungen
				mg	mg%	
25/X	141	alkalisch	1022	31,400	22,270	
26/X	132	,,	,,	30,145	22,496	
27/X	147	,,	,,	31,290	21,286	
28/X	115	,,	1030	23,315	20,274	3 ccm 1% Na-cholatlös. pro Kilo
29/X	131	,,	,,	23,975	18,302	
30/X	154	,,	,,	41,610	27,019	
31/X	166	,,	,,	51,330	30,922	
1/XI	151	,,	,,	71,045	47,049	
2/XI	146	,,	,,	77,435	53,038	

1. *Versuch bei normalem Kaninchen.*

Wie aus den Tabellen I–IV ersichtlichist, beträgt der tägliche Magnesiumgehalt des Harns in der Vorperiode der absoluten Menge nach durchschnittlich 62,79–65,44 mg und prozentual 40,82–44,68 mg %, und in der Versuchsperiode der absoluten Menge nach durchschnittlich 44,69–52,59 mg und prozentual 30,25-34,09 mg %.

Durch Zufuhr von Cholsäure wird die Magnesiumausscheidung im Harn 2 Tage lang sowohl der absoluten Menge nach als auch prozentual ohne Ausnahme herabgesetzt und zwar gegenüber der der Vorperiode der absoluten Menge nach um 18,99–31,71% und prozentual um 16,48–32,29%, während der Magnesiumgehalt im Harn am dritten Injektionstage der absoluten Menge nach durchschnittlich 46,69–54,21 mg und prozentual 31,94–45,16% beträgt und im Vergleich zur Vorperiode der absoluten Menge nach um 13,66–28,64 % und prozentual um 7,48 –27,04 % herabgesetzt wird.

Durch die fortlaufende tägliche Zufuhr von Cholsäure wird die Magnesiumausscheidung im Harn in den späteren Tagen gegenüber der in den früheren Tagen dagegen vermehrt.

Was die Magnesiumausscheidung im Harn in der Nachperiode anbetrifft, so beträgt der Gehalt an Magnesium der absoluten Menge nach durchschnittlich 60,17–76,19 mg und prozentual 43,41 –52,39 mg %, wie aus den Tabellen I–IV ersichtlich ist.

Verglichen nämlich mit dem in der Vorperiode wird der Magnesiumgehalt in 3 Fällen der absoluten Menge nach um 14,56–22,23% und prozentual um 2,71–17,27% vermehrt, im Vergleich zu dem am dritten Tage in der Versuchsperiode der absoluten Menge nach um 27,2–60,78% und prozentual um 23,92–35,90% gesteigert.

Die Magnesiumausscheidung im Harn wird also nach dem Aufhören der Cholatzufuhr im allgemeinen vermehrt oder wieder auf die Norm zurückgebracht.

Aus der Tabelle V ist ersichtlich, dass bei dem fünften Versuch der Magnesiumgehalt in der Vorperiode der absoluten Menge nach durchschnittlich 30,95 mg und prozentual 22,026 mg %, der in der Versuchsperiode absolut 26,67 mg und prozentual 21,87 mg % und der in der Nachperiode absolut 66,6 mg und prozentual 43,67 mg % beträgt. Durch Zufuhr von Cholsäure wird hier auch die Magnesiumausscheidung absolut sowie prozentual herabgesetzt, obwohl bei diesem Versuch der Magnegiumgehalt durch die Verschiedenheit in der Nahrung Okara klein ist, und in der Nachperiode deutlich vermehrt.

Nach der Untersuchung von Iwado(1934) wird durch Zufuhr von Cholsäure der Ca/Mg-Quotient im Organ und Gewebe stark verschoben und ausser in der Lunge und dem Knochen beträchtlich gesteigert.

Wenn einmal eine solch starke Verschiebung im Verhältnis von Calcium zu Magnesium in Organen und Gewebe herbeiführt wird, so muss der Organismus naturgemäss danach streben, das normale Ionengleichgewicht wieder herzustellen,

Diese Daten zeigen hier einen relativen Mangel an Magnesium gegenüber dem Calcium.

Die Mehrausscheidung des Magnesium im Darm bei Zufuhr von Cholsäure nach Kuramoto(1933) kann auch als ein Faktor für diesen Magnesiummangel betrachtet werden.

Hierbei müsste also entweder das Magnesium an dem Gewebe

TABELLE I.

Körpergewicht 2215

Datum (1934)	Harnmenge (ccm)	Reaktion	spec. Gew.	Mg-gehalt		Bemerkungen
				mg	mg%	
12/II	102	alkalisch	1030	77,270	75,755	
13/II	101	„	„	70,443	69,747	
13/II	93	„	„	63,454	68,230	
13/II	76	„	„	57,610	60,011	
16/II	81	„	„	60,888	75,170	
17/II	96	„	„	75,512	78,658	
20/II	Splanchnikotomie					
5/III	105	„	„	39,590	37,705	
6/III	144	„	„	34,403	23,891	
7/III	95	„	„	36,150	38,053	
8/III	114	„	1025	36,368	31,902	
9/III	171	„	„	43,140	25,228	
10/III	96	„	„	53,842	56,086	
11/III	99	„	1030	47,126	47,601	
12/III	96	„	„	28,068	29,237	3 ccm 1% Na-cholatlös. pro Kilo
13/III	74	„	1035	27,468	37,118	
14/III	90	„	„	37,952	42,170	
15/III	117	„	1030	57,720	49,333	
16/III	115	„	„	51,592	62,253	
17/III	124	„	1027	64,000	51,613	
18/III	120	„	„	54,771	45,642	
19/III	106	„	„	66,616	57,185	

TABELLE II.

Körpergewicht 2100

Datum (1934)	Harnmenge (ccm)	Reaktion	spec. Gew.	Mg-gehalt		Bemerkungen
				mg	mg%	
25/II	82	alkalisch	1030	41,993	51,211	
26/II	122	„	„	61,870	50,713	
27/II	92	„	„	48,874	53,124	
28/II	97	„	„	65,584	67,613	
1/III	91	„	„	60,833	66,850	
2/III	92	„	„	42,103	45,763	
7/III	Splanchnikotomie					
19/III	93	„	„	41,939	45,096	
20/III	105	„	„	51,277	48,835	
21/III	100	„	1022	60,504	60,504	
22/III	87	„	1025	49,365	56,741	
23/III	103	„	„	40,245	39,072	
24/III	80	„	1030	23,809	28,761	
25/III	85	„	1022	27,249	32,058	3 ccm 1% Nacholatlös. pro Kilo
26/III	73	„	1025	20,696	28,352	
27/III	88	„	„	26,921	30,593	
28/III	112	„	„	36,751	26,065	
29/III	170	„	„	40,627	23,898	
30/III	125	„	„	55,263	44,210	
31/III	109	„	1022	34,512	31,662	
1/IV	110	„	1020	50,513	45,921	

TABELLE III.

Körpergewicht 2280

Datum (1934)	Harnmenge (ccm)	Reaktion	spec. Gew.	Mg-gehalt		Bemerkungen
				mg	mg%	
4/I	110	alkalisch	1030	83,986	76,351	
5/I	176	„	„	69,133	39,280	
6/I	133	„	„	63,017	47,381	
7/I	158	„	„	74,812	47,350	
8/I	130	„	„	44,771	34,439	
9/I	Splanchnikotomie					
16/I	105	„	„	55,754	53,099	
17/I	126	„	„	60,669	48,134	
18/I	112	„	„	55,536	49,586	
19/I	94	„	„	42,921	45,661	
20/I	106	„	„	51,277	48,372	
21/I	94	„	„	47,399	31,811	3 ccm 1% Na-cholatlös. pro Kilo
22/I	106	„	1035	34,129	56,883	
23/I	112	„	1030	60,233	53,779	
24/I	95	„	„	64,164	67,541	
25/I	107	„	„	47,071	43,992	
26/I	105	„	„	50,839	48,419	
27/I	91	„	„	50,403	55,393	
28/I	78	„	„	36,150	46,347	

TABELLE IV.

Körpergewicht 2150

Datum (1934)	Harnmenge (ccm)	Reaktion	spec. Gew.	Mg-gehalt		Bemerkungen
				mg	mg%	
26/II	106	alkalisch	1030	70,225	67,792	
27/II	95	„	„	72,737	76,565	
28/II	123	„	„	66,785	54,296	
1/III	118	„	„	65,693	55,673	
2/III	116	„	„	59,686	51,451	
7/III	Splanchnikotomie					
19/III	117	„	1027	51,549	44,059	
20/III	124	„	„	51,386	41,440	
21/III	126	„	„	46,634	37,012	
22/III	110	„	„	43,850	39,864	
23/III	113	„	„	36,259	33,888	
24/III	135	„	„	39,098	45,998	
25/III	92	„	1030	31,946	34,724	3 ccm 1% Nacholatlös. pro Kilo
26/III	90	„	„	36,368	40,409	
27/III	145	„	1025	41,829	28,847	
28/III	158	„	„	48,491	30,690	
29/III	130	„	„	35,713	27,472	
30/III	153	„	„	42,593	27,839	
31/III	115	„	„	45,433	39,506	
1/IV	103	„	1030	36,533	35,468	

TABELLE V.

Körpergewicht 2140

Datum (1934)	Harnmenge (ccm)	Reaktion	spec. Gew.	Mg-gehalt		Bemerkungen
				mg	mg%	
12/II	78	alkalisch	1030	77,489	99,344	
13/II	64	„	„	49,365	77,133	
14/II	112	„	„	69,461	62,019	
15/II	92	„	„	65,310	70,988	
16/II	74	„	1035	39,973	54,018	
17/II	83	„	„	55,809	67,239	
20/II	Splanchnikotomie					
5/III	116	„	1030	54,335	46,840	
6/III	116	„	1022	40,627	35,023	
7/III	113	„	1025	45,488	40,255	
8/III	108	„	1030	40,627	37,619	
9/III	103	„	„	48,438	47,027	
10/III	70	„	1035	44,778	63,970	
11/III	71	„	„	43,140	60,760	
12/III	75	„	„	42,373	56,497	3 ccm 1% Na-cholatlös. pro Kilo
13/III	73	„	„	32,273	44,210	
14/III	77	„	1025	28,779	37,375	
15/III	96	„	1035	35,928	37,425	
16/III	140	„	1030	68,314	48,796	
17/III	103	„	„	60,670	58,901	
18/III	104	„	„	55,263	53,138	
19/III	96	„	„	40,814	42,515	

TABELLE VI.

Körpergewicht 2680

Datum (1930)	Harnmenge (ccm)	Reaktion	spec. Gew.	Mg-gehalt		Bemerkungen
				mg	mg%	
4/I	135	alkalisch	1026	91,085	67,471	
5/I	144	„	„	76,014	52,787	
6/I	125	„	1030	65,857	52,685	
7/I	104	„	„	63,657	80,441	
8/I	112	„	„	63,563	56,753	
9/I	Splanchnikotomie					
16/I	102	„	„	56,355	55,250	
17/I	109	„	„	69,406	63,675	
18/I	105	„	1035	59,968	57,113	
19/I	95	„	1030	69,406	73,059	
20/I	96	„	„	78,253	81,513	
21/I	113	„	1035	67,386	59,633	3 ccm 1% Na-cholatlös. pro Kilo
22/I	140	„	„	60,833	43.452	
23/I	114	„	1030	67,253	58,994	
24/I	82	„	„	56,901	69,392	
25/I	83	„	„	58,757	70,792	
26/I	106	„	„	68,202	64,343	

festgehalten werden, um den Ca/Mg-Quotienten möglichst in ein normales Verhältnis zu bringen, oder das Calcium möglichst rasch vom Gewebe herausgebracht werden, indem bei Cholsäurezufuhr das Calcium im Harn vermehrt ausgeschieden wird, wie Sekitoo (1929) es bewiesen hat.

Man darf hierbei noch mit Wahrscheinlichkeit annehmen, dass die Verschiebung des durch Cholsäure angesetzten Magnesium erst nach der Ausscheidung des überschüssigen Calcium in grösseren Massen in die Blutbahn erfolgt, was die Vermehrung des Magnesium im Harn zur Folge hat.

Was den Einfluss des biologischen Vorganges im Kohlehydratoffwechsel, an dem sich das Magnesium als Co-ferment beteiligt, auf die Magnesiumausscheidung im Harn bei Zufuhr von Cholsäure anbetrifft, so muss diese Frage noch weiter verfolgt werden.

2. *Versuch bei splanchnikotomiertem Kaninchen.*

Bei diesem Versuch wurde zuerst die Magnesiumausscheidung im Harn des normalen Kaninchens einige Tage lang bestimmt. Dieses Kaninchen wurde splanchnikotomiert und erst, nachdem es sich von der Operationswunde erholt hatte, zum Versuch gebraucht. Der Versuch wurde in genau der gleichen Weise ausgeführt, wie es beim vorigen Versuch angegeben ist. In der Versuchsperiode wurden diesmal 2 Tage lang je 3 ccm einer 1 %igen Na-Cholatlösung subcutan injiciert.

Während des Versuches wurde das Kaninchen im normalen sowie splanchnikotomierten Zustand immer mit der bestimmten gleichen Nahrung gefüttert.

Aus den Tabellen I–VI ist ersichtlich, dass der Magnesiumgehalt im Harn vor der Operation der absoluten Menge nach durchschnittlich 53,55–76,04 mg und prozentual 48,96–71,79 mg %, dagegen nach der Splanchnikotomie der absoluten Menge nach durchschnittlich 39,58–66,67 mg und prozentual 37,21–66,12 mg %, beträgt. Nur in einem der Fälle ist er etwas vermehrt.

Die Magnesiumausscheidung im Harn des splanchnikotomierten Kaninchens wird also gegen die vor der Splanchnikotomie der absoluten Menge nach durchschnittlich um 12,3–41,4 % und prozentual in 4 Fällen um 16,8–47,8 % vermindert, aber in 2 Fällen um 6,6–8,2 % vermehrt.

Der Magnesiumgehalt des Harns von splanchnikotomierten Kaninchen bei Zufuhr von Cholsäure beträgt der absoluten Menge nach durchschnittlich 23,94–64,11 mg und prozentual 30,21–51,54 mg %, während er in der Nachperiode der absoluten Menge nach durchschnittlich 40,76-62,78 mg und prozentual 31,64-65,88 mg % beträgt; er ist dabei in der Nachperiode gegenüber der Versuchsperiode absolut in einem Fall und prozentual in 2 Fällen vermindert.

Die Magnesiumausscheidung im Harn des splanchnikotomierten Kaninchens bei Zufuhr von Cholsäure wird also gegen die der Vorperiode der absoluten Menge nach durchschnittlich um 3,9-46,2 % und prozentual um 8,1-35,1 % herabgesetzt, aber prozentual in einem Fall um 6,3 % gesteigert.

Die Magnesiumausscheidung im Harn des Kaninchens wird also durch Splanchnikotomie herabgesetzt, und diese Herabsetzung wird durch Cholsäure weiter verstärkt, um dann wieder zum Anfangswert des splanchnikotomierten Kaninchens zurückzukehren.

Die Magnesiumausscheidung im Harn des Kaninchens steht also mit der vegetativen Nervenfunktion bzw. mit der Funktion des sympathischen Nerven in innigem Zusammenhang, ebenso wie es bei der Calciumausscheidung im Harn der Fall war, was von Kitayama (1927) bewiesen wurde.

Beim Ausfall der sympathischen Nervenfunktion wird die Magnesiumausscheidung im Harn vermindert, und diese Verminderung wird durch die den Sympathicus lähmende Cholsäure nach Sekitoo (1930) u. Tsuji (1930) weiter verstärkt.

Diese Tatsache weist darauf hin, dass die durch Zufuhr von Cholsäure verminderte Magnesiumausscheidung im Harn zum Teil auf die Veränderung des Erdalkalienionenverhältnisses in Organ und Gewebe zurückzuführen ist, die durch den Ausfall der sympathischen Nervenerregung verursacht wird.

ZUSAMMENFASSUNG.

1. Die Magnesiumausscheidung im Harn von Kaninchen wird durch Splanchnikotomie herabgesetzt.

2. Die Magnesiumausscheidung im Harn des normalen sowie des splanchnikotomierten Kaninchens wird durch Zufuhr von Cholsäure ebenfalls herabgesetzt.

Aus diesen Daten geht hervor, dass die Magnesiumausscheidung im Harn mit der Funktion der vegetativen Nerven eng verknüpft ist.

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VARIATIONS IN THE ALVEOLAR GASEOUS PRESSURES AS WELL AS THE GASEOUS CONTENTS OF ARTERIAL BLOOD AND ITS pH IN MAN AT REST.

By

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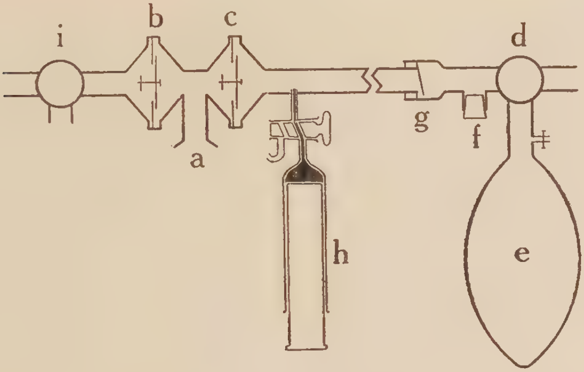
INTRODUCTION.

Studies on the regulation of human respiration which have hitherto been reported are too numerous to be enumerated here. It need only be mentioned that the conception originated by Haldane and Priestley in 1905 that the activity of the respiratory centre is regulated mainly by the CO_2-pressure of the arterial blood, is still a current belief. As the direct determination of the arterial gaseous pressure is not an easy matter, the CO_2-pressure of the alveolar air sample, which is regarded as holding an equilibrium with the arterial blood, is usually taken as its index. Haldane and Priestley concluded from estimations of their alveolar air, on inspiring an air abnormally rich in CO_2, that an increase of 0.2 per cent. of CO_2 in the alveolar air, which is equal to a pressure of ca. 1.5 mm Hg, is sufficient to double the alveolar ventilation. In their monograph published recently (1935), they insisted that this conclusion applies to general cases of human subjects at rest, and also that this variation corresponds to a difference of 0.012 in the blood pH. This latter correspondence they inferred from the experiments of Hasselbalch and Lundsgaard (1912) with human blood. It is, however, questionable if such a minute change in the alveolar CO_2-pressure or in the arterial blood pH so markedly influences the respiratory centre that the lung ventilation is doubled when the subject is respiring atmospheric air. In the example cited in their monograph (pp. 21 & 22) proving the constancy of the alveolar CO_2 of one and the same person during an interval of about 20 months, a variation of from 5.40 to 5.87 % (for J. S. H.) or from 5.985 to 6.835 % (for J. G. P.) are noticeable. They supposed such a daily variation was due to the efforts of the respiratory centre to compensate for various disturbances and thereby to keep the constancy of the blood pH. But the existence of any correlation between the alveolar CO_2-pressure and the arterial blood pH on their daily variations has not yet been experimentally shown. Sasaki (1927), who studied the problem in our Institute, maintained that during normal respiration some other factor than the alveolar CO_2-pressure controls the depth of respiration, which in turn has an influence on the alveolar gaseous pressures.

Recently, in our Institute, Yoshimura (1936), devised a method for estimating the arterial blood pH, and Saito (1937) an apparatus for analysing the arterial blood gases of human subjects, using a few drops of blood collected from an ear-lobe that had been previously warmed so as to dilate the small blood vessels. Now, applying these methods, we outlined a series of researches to examine the regulative action of arterial blood on human respiration. As a preliminary experiment variations of the gaseous contents and the pH of arterial blood were studied in man at rest with reference to their influence upon various respiratory factors.

METHOD AND PROCEDURE.

The arrangement for collecting the expired as well as the alveolar air is sketched in the figure. The subject inspires atmospheric air from a mouth piece (a) through an inspiratory valve (b) and expires through an expiratory one (c). The dead space between the two valves is 37 cc. Expired air may be collected in a rubber bladder (e) of some 14 *l* capacity by turning a three-way tap (d), to which the bladder is attached. The bladder is folded just like a foot-ball bladder when it is empty. Before the expired air is collected in it, it is first washed with expired



air to prevent the sample from being contaminated with the ordinary air remaining in the dead space of the bladder. A side opening (f), which is otherwise left closed, serves for this purpose. The valve (g) serves for counting the respiration. Everywhere in the arrangement the bore is 2 cm. or more except that of the mouthpiece and of the branches of the tap which measures about 1.7 cm. On respiring through this arrangement, the subject feels no resistance, though between the two valves there is a negative pressure of from 0 to -2 mm H_2O during inspiration and a positive one of from 4 to 6 mm H_2O while the expired sample is collected. The air expired during 5 or 10 respirations according to the case is collected in the bladder, while the time lapsed during these respirations is measured with a stop-watch. A measured portion of the sample is analysed by means of the Haldane apparatus. The volume of the rest of it is measured with a laboratory gas-meter graduated to 20 cc. From these observed data are calculated the frequency and the depth of respiration, the lung ventilation, as well as the O_2 absorbed and the CO_2 eliminated. All gaseous volumes are calculated in the dry and N. T. P. state. The gaseous exchange for such a short period is estimated as a function of respiration, but not as an indicator of metabolism of the subject.

The alveolar air is collected in a collection syringe (h) fitted to a side hole of the tube just outside the expiratory valve. The detail of the syringe was described in Sasaki's paper.

Collection of the alveolar air is carried out by the Haldane-Priestly method. The original method is designated to collect the sample twice, once at the end of normal inspiration and next at the end of normal expiration successively and to average the two analyses. According to Bock and Field (1924), however, the CO_2-pressure of the alveolar air taken at the end of expiration approaches more closely that of the arterial blood than that taken at the end of inspiration. So in the present research, alveolar air was collected at the end of normal expiration only.

The procedure of the present research is as follows: The subject respires at rest in a sitting position, through the arrangement described above for 20 minutes or longer. Then his expired air is collected as described above, and his blood is also collected in the manner described in Saito's paper. Immediately after these collections the alveolar air sample is collected by making a sharp and deep expiration at the end of the following normal expiration, while the inspiratory opening is closed by turning an attached tap (i), and the mouth-piece with the tongue.

Throughout the whole research, one of us, Shoji (male, 50 years old, weight 82 kg) who planned the present research, served as the subject. The blood pH was measured by Yoshimura by means of his micro-glass electrode to an accuracy of 0.02 pH. The blood gas analysis was performed by Saito with his micro-modification of the Van Slyke-Neil apparatus. The data are reliable to 1 vol. % at least, Collection of the respiratory samples and their estimates were in charge of Fujimoto. The gas analysis was performed as usual with a Haldane apparatus.

RESULTS.

I. In a single experiment the estimates described above were repeated for about 2 hours with intervals of about 20 minutes. Experiments were repeated several times on different days, and the following facts were found: The blood pH remains unchanged within the scope of experimental accuracy. Variations in the CO_2- or the O_2-content of the blood are within 1.5 vol. %, which somewhat exceeds the scope of experimental error. The alveolar

CO_2-pressure remains quite constant in some cases, but it often shows a sudden change of 1 or 2 mm Hg., which does not always accompany a corresponding variation in the CO_2-content of the arterial blood. The alveolar O_2-pressure is still more labile, varying sometimes 5 mm or so. The respiratory frequency and the depth of a single respiration fluctuate independently of each other, their variations sometimes reaching 20 % of their initial values. These two varying factors rather compensate each other, so that the variation of the lung ventilation per minute never exceeds the mentioned percentage. The largest variation in the gaseous exchange, i. e. the O_2-absorption and the CO_2-elimination per minute, is also of a similar magnitude. A tendency is noticed that the gaseous exchanges estimated as described above vary with the lung ventilation. Table I will suffice as an example. It is especially to be mentioned that no correlation is found between the respiratory depth or the lung ventilation, and the alveolar CO_2-pressure or the CO_2-content of the arterial blood. This shows that the Haldane principle does not apply to the respiratory variations of a man at rest during a few hours.

II. The estimates were repeated at times during the period from 13th May to 1st July, 1936. Results are summarized in Table II. Here we see that the alveolar CO_2-pressure varies from 40.3 to 44.3 mm Hg., and the alveolar O_2-pressure from 94.3 to 105.5 mm Hg. The CO_2- and the O_2-contents of the arterial blood vary from 46.7 to 51.7 vol. % and from 17.1 to 19.8 vol. % respectively, while its pH fluctuates from 7.33 to 7.40. The magnitude of these variations exceeds somewhat the average of those taking place during a few hours. If the blood properties be the same, the gaseous contents of the arterial blood should vary with the alveolar gaseous pressures, and the blood pH should vary relating with its CO_2-content and the alveolar CO_2-pressure according to the Hasselbalch equation. In the table, however, no such correlations are observed, these functions varying quite independently of each other.

Other respiratory functions, i. e. the frequency and the depth of respiration, the lung ventilation and the gaseous exchanges

TABLE I.

Respiration at rest over 2 hours. (Room temp. 16.0–18.5°C)

Time	Alveolar air		Arterial blood			Respiration			Gas. exchange	
	CO_2 mm Hg	O_2 mm Hg	CO_2 vol. %	O_2 vol. %	pH	Freq. /min.	Depth cc.	Ventil. l/min.	CO_2-elim. cc./min.	O_2-abs cc./min
0	44.3	99.2	52.3	19.0	7.35	10.5	357	3.75	144	154
24	44.7	103.6	52.5	20.3	7.34	11.1	—	—	—	—
47	44.5	101.7	51.4	19.1	7.36	10.5	374	3.93	154	151
77	44.9	98.4	52.4	19.2	7.34	9.6	426	4.09	168	191
98	44.5	98.7	52.0	19.0	7.36	10.0	366	3.66	154	167
121	45.7	97.1	51.0	19.4	7.34	10.0	395	3.95	152	167

TABLE II.

Respiration of the same subject at rest on different days.

Date (1936)	Temp. °C	Alveolar air		Arterial blood			Respiration			Gas, exchange	
		CO_2 mm Hg	O_2 mm Hg	CO_2 vol.%	O_2 vol.%	pH	Freq. /min.	Depth cc.	Ventil. l/min.	Co$_2$-elim. cc/min.	O_2-abs. cc/min.
May 13	19.5	42.2	105.5	50.2	19.1	7.35	14.5	440	6.38	225	222
,,	,,	42.2	103.0	51.0	19.4	7.36	15.0	402	6.03	213	216
16	22.0	43.9	100.4	46.7	18.6	7.36	14.6	411	6.00	210	228
,,	,,	41.5	97.6	47.6	19.3	7.36	13.3	439	5.84	189	186
20	19.6	41.0	105.2	50.1	19.4	7.37	11.7	364	4.26	160	169
23	22.6	40.3	94.3	48.0	19.5	7.36	12.2	421	5.14	158	206
27	20.1	40.7	104.7	49.2	18.6	7.36	12.9	481	6.20	220	232
30	20.0	41.7	101.3	50.4	19.8	7.36	11.9	462	5.50	213	244
June 3	22.0	44.3	98.8	51.4	18.3	7.40	11.6	514	5.96	259	290
5	22.7	40.9	103.3	50.9	17.4	7.36	10.9	471	5.13	207	225
8	21.8	42.1	101.6	49.8	18.8	7.35	12.3	450	5.54	218	249
10	22.4	44.0	101.7	50.6	18.6	7.34	11.4	449	5.12	205	235
13	24.9	42.1	103.9	51.7	18.4	7.35	9.9	541	5.36	234	264
16	26.2	44.0	101.6	50.2	18.2	7.33	10.0	486	4.86	218	234
20	28.6	44.1	104.2	48.7	18.4	7.40	9.6	495	4.75	202	203
23	26.9	42.8	101.7	50.0	17.1	7.39	10.6	485	5.14	219	236
July 1	24.8	43.3	99.2	50.1	17.7	7.38	11.6	386	4.48	173	194

per minute, also show quite independent fluctuations, the scopes of which exceed somewhat those occuring over a few hours. It is worth mentioning that the variation in the respiratory depth or the lung ventilation is not controlled by the blood CO_2-content or the alveolar CO_2-pressure, so that the Haldane principle does not apply in this case either.

Correlations, though rather faint, observable among the data given in Table II, are those held between the room temperature and the O_2-content of the blood, as well as the frequency of respiration, the latter two decreasing with raised temperature.

SUMMARY.

The gaseous content and the pH of the arterial blood, the alveolar gaseous pressures, as well as the frequency and the depth of respiration, the lung ventilation and the gaseous exchange per minute were estimated, all at the same time, on one and the same person at rest, repeatedly during a few hours, and also for a period of some seven weeks; and the scope of variations of these functions was studied. No correlations could be found among them. Especially was it ascertained that the Haldane principle, that the alveolar or arterial CO_2 controls the lung ventilation, does not apply in the case of a man at rest during the period mentioned above.

The expenses of this investigation were defrayed partly by a grant fom Hattori-Hokokwai to two of us (R. S. and H. Y.), and partly by another one from the Educational Department of the Government to one of us (K. S.), for which we record our thanks.

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ÜBER DAS VERHALTEN DES CHOLESTERINS UND SEINER ESTER IM BLUTE BEI VERSCHIEDENEN LEBERKRANKHEITEN.

Von

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(Eingegangen am 11. März 1937)

In den letzten Jahren ist das Interesse für das Blutcholesterin wieder stark aufgelebt, und zwar dank der Einführung der Windausschen Digitoninmethode, die in einer relativ geringen Blutmenge die getrennte quantitative Bestimmung des freien und des als Ester gebundenen Cholesterins ermöglicht. Hürthle (1895–96) fand zuerst Oelsäure- und Palmitinsäure-Cholesterinester als normale Bestandteile im Blutserum von Hund, Schwein, Hammel, Rind und Pferd. Heute ist auch beim Menschen die Tatsache bekannt, dass das Blut neben freiem Cholesterin stets eine gewisse Menge als Ester gebundenen Cholesterins enthält. Dabei wurde durch die Untersuchungen mancher Kliniker (Thannhauser und Schaber 1926, Bürger und Habs 1927, Stroebe 1932 u. a.) festgestellt, dass bei stoffwechselgesunden, nicht fiebernden Menschen ungefähr 60–70% resp. 40–50% des gesamten Serum (Plasma)- bezw. Vollblutcholesterins als Estercholesterin vorgefunden wird. Dieses verschiedenartige Verhalten der Esterquote zwischen dem Serum oder Plasma und dem Vollblut ist nicht schwer zu verstehen, da die roten Blutkörperchen fast nur freies Cholesterin enthalten (Wacker und Hueck 1913, 1919).

Nun finden sich in der Literatur zahlreiche Arbeiten über das Verhalten des Blutcholesterins bei Leberkrankheiten. Dabei steht im Brennpunkt unseres Interesses die Frage, ob die Esterquote je nach den Krankheitsformen bestimmte Schwankungen erfährt. Schon wurde Verminderung der Esterwerte von Bürger und Beumer (1913) und Feigl (1918) bei der cholämischen Lipämie gefunden. Bürger (1922) konnte später auch bei Kranken mit Stauungsikterus hinsichtlich der Cholesterinverteilung eine Verminderung der Esterquote feststellen, aber dabei handelte es sich meist gar nicht um eine absolute Verminderung des Estercholesterins, sondern um eine einseitige Vermehrung des freien Cholesterins infolge der Zurückstauung desselben. Bürger glaubte, dass diese eigentümlichen Verhältnisse mit den Störungen der Fettresorption bei gehindertem Gallenabfluss zum Darm in Beziehung gebracht werden könnten, und nahm an, dass für die Veresterung des retinierten freien Cholesterins nicht genügend Fettsäuren zur Verfügung stünden, während nach wiedereinsetzendem Gallenabfluss und Rückkehr der Fettresorptionsgrösse zur Norm die Möglichkeit der Cholesterinveresterung wiederhergestellt sei. In Ergänzung dieser von Bürger erhobenen Befunde haben dann Thannhauser und Schaber (1926) ein Absinken des Estercholesterins, bei manchen Kranken sogar ein vollkommenes Schwinden desselben (Estersturz) beobachten können. Diese augenfällige Veränderung der Relation des Cholesterins zu den Cholesterinestern ging meistens nicht mit einer Vermehrung des Gesamtcholesterins einher. Eine Vermehrung des Gesamtcholesterins konnte nur bei Kranken mit starker Gallenstauung festgestellt werden, während der Estersturz auch bei Zirrhose ohne Ikterus beobachtet wurde. Thannhauser und Schaber brachten diese Verschiebung des Estercholesterins nach der Seite des freien Cholesterins mit einer Schädigung der Rohmann-Schultzschen Cholesterinase bezw. von esterifizierenden Fermenten (Hueck) bei Parenchymerkrankungen der Leber in Zusammenhang. Dagegen behauptete Bürger (1927), dass auch eine absolute Verminderung des Estercholesterins bei normalem Gesamtcholesterinwert durch eine Störung der Fettresorption bezw. des intermediären Fettstoffwechsels hervorgerufen werden kann. Stern und Suchantke (1926) konnten im übrigen in ihren nachprüfenden Untersuchungen eine einigermassen gesetzmässige Verschiebung der Cholesterinverteilung im Serum Leberkranker im Sinne Thannhausers nicht feststellen.

Wenn man weiter experimentelle Untersuchungen in Betracht zieht, so konnten Rosenthal, Licht und Melchior (1926) einen gesetzmässigen Einfluss der Leberexstirpation auf die Cholesterinzusammensetzung des Blutes nicht feststellen, während Enderlen, Thannhauser und Jenke (1926) auch im Blute von leberexstirpierten Hunden bisweilen einen Estersturz beobachteten.

Inzwischen haben Adler und Lemmel (1928) an überaus reichhaltigem klinischem Material ihre Beobachtungen über Cholesterinämie bei Erkrankungen der Leber und Gallenwege mitgeteilt. Im grossen Ganzen wurde bestätigt, dass Gallenstauung zu Hypercholesterinämie führt und Leberparenchymschädigung je nach ihrer Intensität mehr oder weniger starke Verminderung des Cholesterins und gleichzeitig seiner Ester im Blute zur Folge hat.

Nach den gleichartigen Feststellungen verschiedener Autoren der jüngsten Zeit (Mancke 1931, Stroebe 1932, Epstein 1931–1932, Katsura-Tajima-Yoshizumi 1935 und Kamei 1936) tritt zu Beginn eines hepatocellulären Ikterus eine Esterverminderung ein. Während die einmal verminderten Esterwerte unabhängig vom Stande des Gesamtcholesterins regelmässig im Ausheilungsstadium wieder ansteigen, sinken sie beim Fortschreiten des Leberprozesses weiter ab. Aber bei der Leberzirrhose verhalten sich die Cholesterinwerte sehr verschieden, teils unverändert, teils verändert. Ausserdem erschien eine Arbeit von Laroche, Grigaut und Costes (1934), in der bei Kranken mit Ikterus ohne Rücksicht auf seine Ursache und unabhängig von seiner Intensität ein Absinken der Esterquote bis zu 30–40% festgestellt wurde, während die letztere nach ihrer Beobachtung normalerweise 50–70% betrug.

Um nun einerseits die Erscheinung des Estersturzes bei Lebererkrankungen nachzuprüfen und andererseits die widersprechenden Ansichten über die Entstehung des Estersturzes zu kritisieren, stellten wir die vorliegende Arbeit an. Als Untersuchungsmaterial wählten wir solche Patienten, die in die Medizinische Universitätsklinik von Prof. Dr. S. Tsunoo aufgenommen waren und bei denen die klinische Diagnose sichergestellt wurde. Die Blutentnahme wurde immer im nüchternen Zustand ausgeführt.

Das Cholesterin und seine Ester wurden nach der von K. Kusui (1933) vereinfachten gravimetrischen Methode bestimmt. Sie ist gerade für die Reihenuntersuchungen sehr geeignet, da sie auch mit kleinen Blutmengen (5,0 ccm) ausgeführt werden kann. Dabei wurden mit Rücksicht auf die Kranken die Untersuchungen nur im Gesamtblut ausgeführt, da bei der Bestimmung im Serum eine grössere Blutmenge entnommen werden muss.

UNTERSUCHUNGSERGEBNISSE.

I. Der Gehalt an Cholesterin und seinen Estern im Gesamtblute bei gesunden Menschen.

Die physiologischen Werte für Gesamtcholesterin, freies Cholesterin und Cholesterinester im Gesamtblute anzugeben, ist nicht leicht, da sie auch bei Gesunden gewisse Schwankungen zeigen und mit zunehmendem Alter in die Höhe steigen.

Meine Ergebnisse sind in Tabelle I zusammengestellt.

TABELLE I.

Nr.	Name	Alter	Geschlecht	Beruf	Cholosterin im Gesamtblut				
					Datum (1933)	Gesamt g %	Freies g %	Ester g %	Verestert %
1.	S. N.	27.	♂	Laborant	25. I	0,128	0,072	0,056	43.8
2.	K. K.	31.	„	Arzt	20. I	0,140	0,086	0,054	38.5
3.	K. I.	29.	„	„	3. II	0,142	0,074	0,068	47.9
4.	M. T.	23.	♀	Pflegerin	3. II	0,144	0,090	0,054	37.5
5.	K. Y.	20.	♀	„	1. II	0,163	0,092	0,071	43.5
Mittelwert						0,143	0,083	0,060	42.2

Aus der Tabelle ergibt sich, dass das Gesamtcholesterin im Gesamtblute zwischen 0,128 und 0,163 g% variiert und die Ester 37,5–47,9% des Gesamtcholesterins ausmachen. Diese Werte sind etwas niedriger als die von Stroebe (1932), da nach ihm der Gesamtcholesteringehalt im Vollblute zwischen 0,140 und 0,190 g% schwankt, indem die Esterwerte 40–50% des Gesamtcholesterins betragen.

II. Das Verhalten des Cholesterins und seiner Ester im Gesamtblut bei verschiedenen Lebererkrankungen.

Tabelle II zeigt die Resultate, die an 42 Kranken mit Leber- bezw. Gallenwegerkrankungen gewonnen wurden.

In acht Fällen (Nr. 1.–8.) mit sogenanntem Ikterus catarrhalis wurden niemals vermehrte, sondern in einigen Fällen (Nr. 2., 3. und 8.) etwas erniedrigte Gesamtcholesterinwerte konstatiert, während die Esterwerte in allen Fällen gegenüber den normalen deutlich erniedrigt gefunden wurden, so dass die Esterquote zwischen 0 und 25,8% schwankte und 19,2% sich als Mittelwert ergab.

Ein Fall von subakuter gelber Leberatrophie (Nr. 10.), der schon von Kusui und Okabayashi ausführlich mitgeteilt wurde (1935), und ein Fall von Ikterus catarrhalis (Nr. 5.) zeigten ein völliges Verschwinden des Estercholesterins, einen wirklichen Estersturz im Sinne von Thannhauser.

In einem Falle von Salvarsanikterus (Nr. 9.) wurde zu Beginn der Erkrankung eine beträchtliche Erniedrigung des Gesamtcholesterins (0,085 g%) und der Esterquote (16,4%) nachgewiesen.

Wir konnten bei vier Fällen (Nr. 11.–14.) von Ikterus infectiosus (Leptospirosis icterohämorrhagica) die Cholesterinwerte bestimmen. In allen Fällen nahmen die Ester gegenüber der Norm absolut und auch relativ im Verhältnis zum Gesamtcholesterin stark ab. In je zwei Fällen wurde eine leichte Verminderung bezw. Steigerung des Gesamtcholesterins beobachtet.

Bürger und Habs haben bei einer Reihe von Leberzirrhotikern das Verhältnis vom freien zum veresterten Cholesterin im Serum einer Untersuchung unterzogen. Sie haben dabei ihre Fälle in 2 Gruppen—ikterische und anikterische—geteilt. Die Fälle ohne Blutikterus zeigten normale prozentuelle Esterwerte, dabei war meistens das Gesamtcholesterin beträchtlich vermehrt, so dass auch die absoluten Esterwerte weit über der Norm lagen. In der Gruppe mit Blutikterus allein fanden sich Fälle mit verminderten prozentuellen Esterwerten. Hier fanden sich auch Fälle, in welchen die Gesamtcholesterinwerte normal waren, woraus

TABELLE

Nr.	Name	Alter	Geschlecht	klinische Diagnose	Cholesterin im Gesamtblut					
					Datum	Gesamt g%	Freies g%	Ester g%	Verestert %	Datum
1	I. S.	21	♂	Ikterus catarrhalis mit	17 IX (32)	0.137	0.104	0.033	24.1	17 IX
				Lues II Stadium	28 IX („)	0.149	0.103	0.046	30.9	28 „
2	J. S.	38	♂	Ikterus catarrhalis	26 IX („)	0.118	0.100	0.018	15.2	26 „
					5 X („)	0.142	0.097	0.045	31.7	5 X
					9 XI („)	0.146	0.084	0.062	42.5	9 XI
3	Y. F.	22	♂	„ „	26 IX („)	0.120	0.089	0.031	25.8	26 IX
					9 X („)	0.143	0.100	0.043	30.1	9 X
4	M. T.	18	♂	„ „	9 X („)	0.132	0.100	0.032	24.2	9 X
5	M. N.	45	♂	„ „	27 XII („)	0.154	0.154	0	0	27 XII
6	A. U.	39	♂	„ „	14 I (33)	0.124	0.097	0.027	21.8	14 I
7	S. N.	25	♂	„ „	8 II („)	0.125	0.098	0.027	22.4	8 II
					13 II („)	0.133	0.109	0.024	18.0	13 „
					24 II („)	0.166	0.093	0.073	43.9	24 „
8	I. K.	17	♀	„ „	23 VIII („)	0.113	0.085	0.028	24.8	23 VIII
					29 „ („)	0·187	0.111	0.076	39.9	29 „
9	Y. W.	25	♂	Salvarsanikterus	24 VII (32)	0.085	0.071	0.014	16.4	24 VII
					30 „ („)	0.154	0.095	0·059	38.3	30 „
					6 VIII („)	0.160	0·087	0.073	45.6	6 VIII
					11 „ („)	0.156	0.077	0.079	50.6	11 „
					1 IX („)	0.197	0.113	0.084	42.7	1 IX
10	T. H.	46	♀	Subakute gelbe	15 IV (33)	0.142	0.142	0	0	15 IV
				Leberatrophie	20 „ („)	0.149	0.148	0.001	0.7	20 „

II (1).

Serumbilirubin			Harnbefunde					Kotbefunde		Bemerkungen
Hiymans van den Bergh		Kumegawa mg %	Datum	Gmelinsche Probe	Haysche Probe	Urobilin	Urobilinogen	Datum	Aussehen etc.	
direkte Reaktion	indirekte Reaktion mg %									
prompt	3.50	4.00	17 IX	(⧻)	(±)	(⧺)	(−)	17 IX	normal	Wa R (⧻)
,,	1.05	3.30	26 IX	(±)	(−)	(−)	(„)	—	—	ausgeheilt
,,	7.85	16.14	,, ,,	(⧺)	(+)	(„)	(„)	26 IX	normal	
,,	5.32	14.56	—	—	—	—	—	—	—	
verzögert	0.61	—	—	—	—	—	—	—	—	ausgeheilt
prompt	8.12	21.60	26 IX	(⧺)	(+)	(−)	(−)	26 IX	acholisch	
,,	1.21	—	—	—	—	—	—	9 X	gelblich braum	augeheilt
,,	1.78	3.18	9 X	(±)	(−)	(⧻)	(⧻)	9 X	normal	,,
,,	14.29	30.86	27 XII	(⧻)	(+)	(+)	(−)	27 XII	acholisch	Prognose unklar
,,	6.90	14.60	14 I	(⧻)	(±)	(„)	(„)	14 I	leicht acholisch	ausgeheilt
,,	6.42	12.27	8 II	(⧻)	(⧻)	(−)	(„)	8 II	acholisch Sublimatprobe (+)	
,,	7.86	16.52	13 ,,	(⧻)	(„)	spur	(+)	13 ,,	acholisch Sublimatprobe (+)	
,,	0.75	4.05	24 ,,	(±)	(−)	(-)	(−)	24 ,,	normal	ausgeheilt
,,	7.70	11.52	23 VIII	(+)	(„)	(„)	(„)	23 VIII	,,	
,,	0.86	2.65	29 ,,	(−)	(„)	(⧻)	(+)			ausgeheilt
,,	6.57	15.43	24 VII	(⧻)	(+)	(+)	(−)	24 VII	acholisch Sublimatprobe (−)	
,,	6.96	12.54	30 ,,	(„)	(„)	(„)	—	30 ,,	,, ,,	
,,	5.82	12.78	6 VIII	(⧺)	(−)	—	(−)	6 VIII	bräunlich gelb.	
,,	6.43	10.61	11 ,,	(⧻)	(+)	(+)	(„)	11 ,,	acholisch Sublimatprobe (−)	
,,	2.70	10.60	1 IX	(„)	—	(„)	(+)	1 IX	gelblich	ausgeheilt
,,	19.23	22.56	15 IV	(„)	(−)	(„)	(„)	15 IV	weich, Sublimatprobe grünlich	
,,	12.86	26.86	23 ,,	(„)	(+)	(⧺)	(⧺)	23 ,,	Sublimatprobe rötlich	am 28. IV (33) gestorben

TABELLE

Nr.	Name	Alter	Geschlecht	klinische Diagnose	Cholesterin im Gesamtblut					
					Datum	Gesamt g%	Freies g%	Ester g%	Verestert %	Datum
					26 IV (33)	0.112	0.103	0.009	8.0	26 IV
11	M. F.	45	♂	Leptospirosis	23 VIII („)	0.199	0.179	0.020	10.0	23 VIII
12	K. M.	56	♂	icterohaemorrhagica	23 VIII (35)	0.193	0.165	0.028	14.5	„ „
13	T. K.	47	♂	„ „	9 X („)	0.129	0.117	0.012	9.2	9 X
14	H. S.	34	♂	„ „	28 I (36)	0.124	0.107	0.017	13.7	28 I
15	Y. Y.	19	♂	haemolytischer Ikterus	26 X (34)	0.147	0.085	0.062	42.2	26 X
					16 VI (35)	0.144	0.082	0.062	43.0	16 VI
16	M. T.	24	♂	„ „	19 VIII („)	0.126	0.076	0.050	39.9	19 VIII
					23 „ („)	0.129	0.075	0.054	39.9	2 IX
17	K. S.	49	♂	chron. Alkoholismus mit beginnender Leberzirrhose	27 VI (32)	0.095	0.071	0,024	25.2	27 VI
18	H. S.	38	♂	biliäre Leberzirrhose	24 III (33)	0.133	0.108	0.025	18.8	24 III
					13 IV („)	0.137	0.108	0.029	20.4	13 IV
					10 V („)	0.124	0.097	0.027	21.7	10 V
					31 V („)	0.103	0.083	0.020	20.4	31 V
19	K. T.	46	♂	atrophische Leberzirrhose	27 VII („)	0.163	0.100	0.063	38.6	27 VII
20	G. K.	59	♂	„ „	27 „ („)	0.144	0.096	0.048	33.3	25 VII
21	Y. Y.	34	♂	„ „	12 VI (35)	0.195	0.155	0.040	20.6	12 VI
22	I. M.	45	♂	Leberzirrhose, entwickelt aus subakuter gelber Leberatrophie, welche nach Salvarsaninjektion entstand.	15 VII („)	0.127	0.103	0.024	18.8	12 VII
					21 VIII („)	0.156	0.104	0.052	33.3	23 VIII
23	T. A.	69	♂	atrophische Leberzirrhose	16 IX („)	0.166	0.116	0.050	30.1	16 IX
24	S. T.	27	♂	Leberkrebs	9 VI (32)	0.136	0.079	0.057	42.0	9 VI
25	T. U.	66	♂	„	8 VII (33)	0.293	0.217	0.076	25.8	8 VII

II (2).

Serumbilirubin			Harnbefunde					Kotbefunde		Bemerkungen
Hijmans van den Bergh		Kumegawa mg %	Datum	Gmelinsche Probe	Haysche Probe	Urobilin	Urobilinogen	Datum	Aussehen etc.	
direkte Reaktion	indirekte Reaktion mg %									
prompt	17.14	30.46	26 IV	(+++)	(+)	(+)	(++)	23 IV	bräunlich gelb.	Sektion !
„	27.80	50.00	23 VIII	(„)	(++)	(++)	(+)	23 VIII	normal	am 27 VIII (33) gestorben
„	8.28	12.31	„ „	(++)	(+)	(+)	(−)	„ „	schleimig eitrig	am 28 VIII (35) gestorben
„	15.72	22.14	9 X	(+++)	(„)	(„)	(+)	9 X	normal	am 16 X (35) gestorben
„	17.60	28.80	28 I	(„)	(„)	(−)	(„)	28 I	„	am 13 II (36) gestorben
verzögert	4.28	5.06	23 X	(−)	(−)	(++)	(„)	23 X	„	
„	5.50	6.28	16 VI	(„)	(„)	(„)	(++)	16 VI	„	arbeiten !
„	4.07	5.35	19 VIII	(„)	(„)	(+++)	(„)	19 VIII	„	
„	3.86	5.35	23 „	(„)	(„)	(„)	(„)	—	—	arbeiten !
„	unmessbar (++)	—	27 VI	(„)	(„)	(−)	(−)	27 VI	normal	Prognose unklar
prompt	9.63	16.05	24 III	(+++)	(„)	(+)	(„)	24 III	„	
„	2.43	7.33	13 IV	(++)	(„)	(„)	(„)	10 IV	„	
„	2.14	6.94	10 V	(+++)	(„)	(„)	(„)	10 V	„	am 14 VII (33) gestorben
„	1.78	5.68	31 V	(++)	(++)	(+++)	(+++)	31 „	„	Sektion !
verzögert	0.92	1.06	27 VII	(−)	(−)	(+)	(+)	27 VII	„	am 2 X (33) gestorben
II phasisch prompt	1.46	2.02	„ „	(„)	(+)	(++)	(−)	26 „	„	am 20 IX (33) gestorben
prompt	17.10	30.85	12 VI	(+++)	(+++)	(−)	(„)	12 VI	„	
„	4.64	11.57	12 VII	(++)	(−)	(„)	(„)	12 VII	„	Ascites (+)
II phasisch prompt	0.85	1.44	23 VIII	(−)	(„)	(+)	(„)	23 VIII	„	Ascites (−) Am 22 IX (35) gebessert entlassen
prompt	5.19	9.20	16 IX	(++)	(+)	(++)	(+)	16 IX	acholisch Sublimatprobe (−)	
prompt (?)	0.62	—	9 VI	(−)	(−)	(−)	(−)	9 VI	normal	
prompt	11.36	19.20	8 VII	(+++)	(+)	(„)	(„)	6 VII	normal Okkultes Blut (++)	

Tabelle

Nr.	Name	Alter	Geschlecht	klinische Diagnose	Cholesterin im Gesamtblut					Datum
					Datum	Gesamt g %	Freies g %	Ester g %	Verestert %	
26	I. I.	45	♂	Leberabszess	12 VI (32)	0.148	0.084	0.064	43.2	12 VI
27	M. Y.	48	♂	„	23 VIII (33)	0.108	0.083	0.025	23.1	23 VIII
28	M. K.	35	♂	„	16 IX („)	0.143	0.105	0.038	26.6	16 IX
29	M. M.	55	♂	„	13 „ (34)	0.157	0.135	0.022	14.0	13 IX
30	O. K.	48	♂	Chronischer Ikterus von unklarer Natur	1 VI („)	0.142	0.094	0.048	33.8	1 VI
31	H. Y.	37	♂	Gallensteinkrankheit	22 „ (32)	0.135	0.084	0.051	37.7	22 VI
32	M. I.	43	♀	„	28 VII („)	0.085	0.075	0.010	11.8	28 VII
33	H. Y.	41	♂	„	3 IX („)	0.132	0.095	0.037	28.0	3 IX
34	K. N.	43	♂	Gallensteinkrankheit mit Gallenweg-Duodenalfistel	24 VIII („)	0.146	0.097	0.049	33.5	24 VIII
					6 IX („)	0.149	0.094	0.055	36.2	6 IX
35	Y. K.	28	♂	Gallensteinkrankheit mit Gallenweg-Duodenalfistal	10 II (33)	0.108	0.078	0.030	27.7	10 II
36	T. H.	64	♂	Gallensteinkrankheit	13 IV („)	0.225	0.130	0.095	42.2	13 IV
				„	20 „ („)	0.213	0.133	0.080	37.5	20 „
37	H. J.	22	♂	„	13 IX (34)	0.202	0.142	0.060	30.0	13 IX
38	H. N.	55	♂	„	25 VIII (33)	0.165	0.112	0.053	32.1	25 VIII
39	I. A.	44	♂	Staungsikterus durch Pankreastumor	5 I („)	0.249	0.191	0.058	23.2	5 I
					18 I („)	0.298	0.248	0.050	16.8	18 I
40	S. M.	42	♂	Totaler Verschluss des Ductus choledochus	21 X („)	0.361	0.283	0.078	21.7	21 X
41	I. K.	59	♂	Verschluss des Ductus choledochus (Krebs?)	15 VII (35)	0.231	0.203	0.028	12.1	15 VII
42	M. S.	42	♀	Gallenblasenkrebs (Anamnese Cholelithiasis)	15 VI (32)	0.142	0.081	0.061	42.9	15 VI

II (3).

Serumbilirubin			Harnbefunde					Kotbefunde		
Hiymans van den Bergh										
direkte Reaktion	indirekte Reaktion mg %	Kumegawa mg %	Datum	Gmelinsche Probe	Haysche Probe	Urobilin	Urobilinogen	Datum	Aussehen etc.	Bemerkungen
prompt	0.61	—	12 VI	(−)	(−)	(+++)	(−)	12 VI	normal	
II phasisch prompt	1.00	1.75	23 VIII	(„)	(„)	(+)	(„)	23 VIII	„	chirurgische Operation !
verzögert	0.64	0.96	16 IX	(„)	(„)	(„)	(„)	16 IX	„	am 10 X (33) gestorben, Sektion!
prompt	5.71	18.08	13 IX	(++)	(+)	(„)	(+)	13 „	acholisch	
II phasisch verzögert	1.56	3.86	1 VI	(+)	(±)	(+++)	(„)	1 VI	normal	Wa-R (−) Murata (±)
prompt	7.07	13.02	22 VI	(++)	(++)	(−)	(−)	22 „	„	am 22 VI (32) Anfall
„	1.96	2.84	28 VII	(−)	(−)	(+)	(+)	28 VII	„	Pause
„	1.43	2.48	3 IX	(±)	(„)	(−)	(−)	3 XI	„	am 2 IX (32) leichter Anfall
„	1.10	—	24 VIII	(−)	(+)	(++)	(„)	24 VIII	„	chirurgische Operation!
„	0.96	2.26	—	—	—	—	—	—	—	gebessert entlassen chirurg. Operation
prompt (?)	unmessbar (+)	0.57	10 II	(−)	(−)	(−)	(−)	10 II	normal	gebessert entlassen
prompt	1.42	—	13 IV	(„)	(+)	(++)	(+)	13 IV	„	
„	0.71	2.12	20 „	(„)	(−)	(„)	(„)	20 IV	„	
„	30.70	48.20	13 IX	(+++)	(+)	(−)	(−)	13 IX	„	
„	unmessbar (+)	1.02	25 VIII	(−)	(−)	(+)	(+)	18 VIII	„	Pause
„	5.00	—	5 I	(+++)	(++)	(−)	(−)	5 I	acholisch Sublimatprobe (−)	chirurg. Operation gebessert entlassen
„	3.91	7.71	18 I	(„)	—	(„)	—	18 „		am 2 XI (33) gestorben
„	20.70	38.60	21 X	(„)	(+)	(+)	(+)	21 X	acholisch Sublimatprobe (−)	Sektion! Wa-R (−)
„	12.34	31.74	7 VII	(„)	(++)	(„)	(„)	7 VII	diarrhoisch acholisch	
„	0.72	1.32	15 VI	(−)	(−)	(−)	(−)	15 VI	normal	chirurg. Operation am 14 X (32) gestorben

TABELLE

Nr.	Name	Alter	Geschlecht	klinische Diagnose	Cholesterin im Gesamtblut					Blutdruck (mm Hg)	
					Datum	Gesamt g%	Freies g%	Ester g%	Verestert %	Max.	Min.
1	W. T.	60	♀	essentielle Hypertonie	9 VI (32)	0.136	0.088	0.047	34.7	220	100
2	S. N.	42	„	essentielle Hypertonie mit Aortenstenose	6 VII (32)	0.145	0.074	0.071	49.0	150	80
3	S. M.	47	„	essentielle Hypertonie	10 V (33)	0.162	0.094	0.068	41.9	215	130
4	K. M.	48	„	„ „	„ „ „	0.187	0.108	0.079	42.2	190	105
5	T. K.	67	„	essentielle Hypertonie mit Diabetes mellitus	„ „ „	0.185	0.100	0.085	45.9	220	100
6	K. K.	41	♀	essentielle Hypertonie	9 V (35)	0.192	0.109	0.083	43.2	240	160
					6 VI („)	0.189	0.114	0.075	39.7	226	142
7	S. T.	31	♂	Schrumpfniere	25 V (32)	0.166	0.088	0.078	47.2	210	140
					6 VII (32)	0.131	0.065	0.066	50.4	215	140
				(mit starker Anämie)	24 „ („)	0.076	0.047	0.029	37.7	225	150
8	S. T.	59	♂	Schrumpfniere	26 VI („)	0.145	0.076	0.069	47.2	240	150
9	S. T.	45	„	„	16 V („)	0.143	0.074	0.069	47.3	230	110
10	A. F.	52	♀	„	6 VII („)	0.143	0.082	0.061	42.6	240	110
11	K. M.	35	„	chronische Nephritis	22 VI („)	0.121	0.076	0.045	37.2	130	85
12	T. O.	50	♂	chron. Nephritis mit Lues	1 VII („)	0.145	0.081	0.061	41.2	120	80
13	T. Y.	24	♀	chron. Nephritis	23 V (35)	0.245	0.149	0.096	39.2	125	70
14	U. M.	43	♀	chron. Nephritis mit Mitralinsuffizienz	13 III („)	0.229	0.141	0.088	38.4	220	110
15	S. W.	22	♂	Lipoidnephrose	16 X (32)	0.383	0.216	0.167	43.6	125	85
16	T. O.	59	♀	Starke Anämie (Ankylostomiasis)	7 XI („)	0.092	0.061	0.031	33.6	—	—
17	S. O.	52	♀	Invagination in der Ileocaecalgegend	2 II (33)	0.158	0.100	0.058	36.7	—	—

III.

Reststickstoff im Blut		Nierenfunktionsprüfung (Wasserversuch)			Harnbefunde					Bemerkungen
Datum	mg %	Datum	Verdünnungsvermögen	Konzentrationsvermögen	Datum	Lipoid	Zylinder	Eiweiss	Erythrocyten	
17. VI	54.0	17. VI	normal	normal	9. VI	(–)	(–)	(–)	(–)	
9. VII	26.4	—	—	—	9. VII	(„)	(„)	(„)	(„)	
10. V	42.8	7. V	normal	normal	10. V	(„)	(„)	(„)	(„)	
„ „	33.2	6. V	„	„	10. V	(„)	(„)	(„)	(„)	
„ „	43.7	—	—	—	10. V	(„)	(„)	(„)	(„)	Zucker im Harn (+++)
19. V	40.0	28. I (35.)	nicht so schlecht	nicht so schlecht	19. V	(„)	(„)	0.5 ‰	(+)	
3. VI	36.0	—	—	—	5. VI	(„)	(„)	1.5 ‰	(+)	
8. VI	57.4	4. V	normal	normal	25. V	(++)	(„)	4.0 ‰	(+)	
29. VI	58.6	—	—	—	6. VII	(+)	(+)	10 ‰	(+)	Am 28. VII (32.) gestorben Sektion !
25. VII	71.7	–	—	—	21. VII	(++)	(+)	5 ‰	(+)	
26. VI	43.6	30. VI	schlecht	schlecht	26. VI	(–)	(+)	2 ‰	(++)	
16. V	86.8	Wegen schlechtes Zustandes konnte nicht ausführen			16. V	(–)	(–)	2 ‰	(–)	
6. VII	46.0	6. VII	normal	normal	6. VII	(–)	(–)	(+)	(+)	
29. VI	31.5	29. VI	„	„	22. VI	(+)	(+)	4 ‰	(+)	
1. VII	71.0	29. VI	„	„	1. VII	(+)	(–)	0.5 ‰	(–)	Wa-R (+++)
27. V	32.0	1. VII	nicht so schlecht	nicht so schlecht	23. V	(++)	(+)	1.8 ‰	(+)	
13. III	27.5	—	—	—	13. III	(+)	(+)	3.5 ‰	(+)	
10. X	50.7	—	—	—	13. X	(++)	(–)	45 ‰	(–)	Am 19. X (32.) gestorben (Pneumokokkenperitonitis)
—	—	—	—	—	7. XI	(–)	(–)	(–)	(–)	Sahli 21, Erythrocytenzahl 2, 80 Million Wa-R (++)
—	—	—	—	—	2. II	(–)	(–)	(–)	(–)	chirurgische Operation ausgeheilt entlassen

also auch eine Verminderung der absoluten Esterwerte resultierte. **Adler** und **Lemmel** fanden bei den Formen der Zirrhose, die aus akuter gelber Atrophie hervorgegangen waren, meist normale oder etwas verminderte Cholesterinwerte, während bei den Fällen von alkoholischer Leberzirrhose sich immer mehr oder weniger starke Cholesterinvermehrung fand. Aber die Ester pflegten nicht wesentlich von der Norm abzuweichen. Nach den **Stroebe**schen Erfahrungen war nicht anzunehmen, dass das von obigen Autoren angegebene verschiedene Verhalten des Blutcholesterins auf den verschiedenen Formen der Leberzirrhose beruhte.

Wir fanden in 6 (Nr. 17., 18., 20.–23.) unter 7 Fällen (Nr. 17.–23.) von Leberzirrhose eine Herabsetzung der Esterquote unter die Norm, dabei waren die Gesamtcholesterinwerte normal oder subnormal (Nr. 17.), woraus also auch eine absolute Verminderung des Estercholesterins resultierte.

Ausserdem wurde bei 4 (Nr. 27.–30.) von 7 Kranken (Nr. 24.–30.) mit Leberparenchymschädigung von anderen verschiedenen Ätiologien (Leberkrebs, Leberabszess und chronischer Ikterus von unklarer Natur) eine absolute Abnahme des Estercholesterins bei normalem Gesamtcholesteringehalt, nur in einem Fall (Nr. 25.) eine mit einer Vermehrung der Gesamtcholesterinmenge verbundene relative Verminderung der Esterquote trotz einem normalen Werte des absoluten Estercholesterins und in zwei Fällen (Nr. 24. und 26.) ganz normale Verhältnisse festgestellt.

Die oben beschriebenen Resultate lassen deutlich erkennen, dass die Schädigung der Leberzellen sehr häufig von einer Verminderung des Gesamtcholesterins und besonders einer Abnahme der Cholesterinester im Blute begleitet wird.

In manchen Fällen von hepatozellulären Ikterus stellten wir Cholesterinbestimmungen in verschiedenen Krankheitsstadien wiederholt an, wie es schon von **Epstein**, **Stroebe** und **Kamei** versucht wurde.

In 5 Fällen von Ikterus catarrhalis (Nr. 1., 2., 3., 7. und 8.) und in 1 Fall von Salvarsanikterus (Nr. 9.) wurde zu Beginn der Erkrankung als Regel Erniedrigung der Esterquote und bisweilen auch Abnahme des Gesamtcholesterins konstatiert. Mit fortschreitender Besserung kehrten beide Werte allmählich zu normalen oder übernormalen Werten zurück, während die verminderte Esterquote in je einem Fall von subakuter gelber Leberatrophie (Nr. 10.) und von biliärer Leberzirrhose (Nr. 18.), die beide letal endeten, im ganzen Verlauf bestehen blieb und sogar vor dem Tode sich die Gesamtcholesterinwerte auch subnormal erniedrigten.

Also scheint eine absolute Verminderung des Estercholesterins bei normalem bezw. subnormalem Gesamtcholesterinwert für schwere Leberschädigung zu sprechen. Daher glauben wir, dass in der Klinik einer ausführlichen Reihenuntersuchung des Cholesterins und seiner Ester eine wichtige Bedeutung für die Prognosestellung der Lebererkrankung zukommt.

Wir beobachteten weiter die Veränderungen des Blutcholesterins auch in 12 Fällen (Nr. 31.–42.) von Gallenwegerkrankungen. In zwei Fällen (Nr. 31. und 42.) fand sich ein ganz normales Verhalten für das Gesamt- und Estercholesterin. Starke Vermehrung des Gesamtcholesterins fanden wir in 5 Fällen (Nr. 36., 37., 39.–41.), unter denen 4 Fälle (Nr. 37., 39., 40. und 41.) eine Verminderung der Esterquote aufwiesen, wenn auch die absoluten Esterwerte mit Ausnahme eines einzigen Falles (Nr. 41.) ganz normal waren. Da es angenommen wird, dass die Leber des Menschen im physiologischen Zustand nur freies Cholesterin in die Galle ausscheidet, so ist es möglich, dass eine mechanische Verstopfung der extrahepatischen Gallenwege mit einer einseitigen Vermehrung des freien Cholesterins im Blute einhergeht. Ein anderer Fall (Nr. 36.) mit einer beträchtlichen Vermehrung des Gesamtcholesterins zeigte, zweimal untersucht, immer normale Esterquote, so dass auch die absoluten Esterwerte weit über der Norm lagen. Andere 5 Fälle (Nr. 32.–35. und 38.) zeigten ein deutliches Absinken der Esterquote gegenüber der Norm, während die Gesamtcholesterinwerte normal oder subnormal standen. In solchen Fällen brachte vielleicht eine sekundäre Störung der Leberfunktion infolge Gallenstauung die Verminderung der Esterquote hervor.

Aus diesem Ergebnissen ergibt sich, dass das Cholesterin und seine Ester im Blute bei den Gallengangerkrankungen je nach dem Grad der Gallenwegsstörungen und der Krankheitsphase verschiedenes Verhalten zeigen. Die alte Ansicht der französischen Schule, dass die Cholelithiasis immer von einer Hypercholesterinämie begleitet wird und diese Hypercholesterinämie eine Disposition oder Diathese für die Cholelithiasis darstellt, kann ich ebenso wie **Gardner** (1932) nicht teilen.

Was den hämolytischen Ikterus betrifft, fanden **Adler** und **Lemmel** in 8 Fällen die Gesamtcholesterinwerte zwischen 70 und 140 mg%, allerdings mit colorimetrischer Methode. Nach **Bürger** verhält sich die Esterquote beim hämolytischen Ikterus wie in der Norm. In zwei einschlägigen Fällen (Nr. 15. und 16.) fanden wir normale Werte des Cholesterins und seiner Ester im Blute.

III. Das Verhalten des Cholesterins und seiner Ester im Blute bei Krankheiten von anderen Organen als der Leber.

Als Kontrolle haben wir das Blut von anderen als Leberkranken auf freies und Estercholesterin untersucht.

Die Resultate sind in Tabelle III zusammengestellt.

Unter 6 Fällen (Nr. 1.–6.) von essentieller Hypertonie zeigten 3 Fälle (Nr. 4.–6.) leichte Vermehrung des Gesamtcholesterins, während die Esterquote sich in 5 Fällen normal verhielt und nur in einem Fall (Nr. 1.) eine leichte Verminderung erfuhr.

In 4 Fällen (Nr. 7.–10.) von Schrumpfniere und 2 Fällen (Nr. 11. und 12.) von chronischer Nephritis fanden wir normale Werte für das Cholesterin und seine Ester.

Während **Hueck** (1925) eine Hypocholesterinämie bei Anämie beobachtete, fanden wir auch in Fällen von Anämie infolge Schrumpfniere (Nr. 7.) resp. Ankylostomiasis (Nr. 16.) ein Absinken des Gesamtcholesterinwerts; dabei verminderte sich die Esterquote nicht erheblich.

In einem Fall von Invagination in der Ileocäcalgegend (Nr. 17.), die chirurgisch später reseziert wurde, verhielt sich der Cholesterinspiegel normal.

Bemerkenswert ist, dass ein Fall (Nr. 15.) von Lipoidnephrose und 2 Fälle (Nr. 13. und 14.) von chronischer Nephritis mit exzessiver Vermehrung des Gesamtcholesterins (0,383, 0,245 und

0,229 g%) eine normale Esterquote zeigten.

IV. Tierversuche.

Da unsere oben geschilderten Resultate ein regelmässiges Verhalten der Esterquote bei der Leberparenchymschädigung einerseits und eine weniger einheitliche Veränderung der relativen Esterwerte bei der Passagestörung der Gallenwege durch Gallenstein bezw. verschiedene andere Momente andererseits erkennen liessen, so schien es uns wünschenswert, im Tierversuche zu verfolgen, wie sich bei toxischer Leberschädigung und Unterbindung der Ducti choledochus und cysticus das Cholesterin und seine Ester im Blut verhalten.

Wir wählten Hunde als Versuchstiere.

A. Die Schwankung des Cholesteringehaltes im normalen Hundeblute im Verlauf von 24 Stunden.

Das Blut von 2 normalen Hunden wurde auf freies Cholesterin und Estercholesterin in Abständen von 5, 10 und 24 Stunden nach der ersten Blutentnahme im nüchternen Zustand am frühen Morgen untersucht.

Die Resultate sind folgende (Tabelle IV).

Der Gesamtcholesteringehalt schwankte in einem Falle (Nr. 1.) zwischen 0,110 und 0,120 g% und in einem anderem Falle (Nr. 2.) zwischen 0,124 und 0,132 g%, wobei die Esterwerte 40,0–42,2% resp. 39,4–42,8% des Gesamtcholesterins betrugen. Es ergibt sich also, dass die Tagesschwankung des Cholesteringehaltes im normalen Hundeblut ca. 10% beträgt, und dass die Esterquote fast keine nennenswerte Schwankung zeigt.

B. Das Verhalten des Cholesterins und seiner Ester im Hundeblute bei Toluylendiaminvergiftung.

Toluylendiamin (Merck) wurde als 2,5%ige Lösung in der Dosis von 0,05–0,07 g pro Kilo Körpergewicht 3 weiblichen Hunden subkutan injiziert und das Blut der Hunde wurde auf freies und Estercholesterin, gleichzeitig auf Serumbilirubin untersucht.

Die Resultate sind in Tabelle V zusammengestellt.

TABELLE IV.

Hund	Blutentnahme		Cholesterin im Gesamtblut			
	Datum (1933)	Stundenzahl nach der ersten Blutentnahme	Gesamt g %	Freies g %	Ester g %	Verestert %
Nr. 1. ♂ 10.6 Kg	8^{h}am10. II	erstes Mal	0.113	0.067	00.46	40.7
	1^{h}pm „ „	5	0.120	0.072	0.048	40.0
	6^{h}pm „ „	10	0.116	0.067	0.049	42.2
	8^{h}am11. II	24	0.110	0.064	0.046	41.8
Nr. 2. ♀ 12.5 Kg	8^{h}am17. II	erstes Mal	0.132	0.080	0.052	39.4
	1^{h}pm „ „	5	0.126	0.074	0.052	41.2
	6^{h}pm „ „	10	0.130	0.076	0.054	41.6
	8^{h}am18. II	24	0.124	0.071	0.053	42.8

Die Gesamtcholesterinmenge begann 5 (Nr. 3.)—10 Stunden (Nr. 1. und 2.) nach der Injektion zuzunehmen und erreichte den maximalen Wert nach (Nr. 1.), 7 (Nr. 2.) resp. 10 (Nr. 3.) Tagen. Bei allen Versuchshunden wurde die absolute Verminderung des Estercholesterins schon 5 Stunden nach der Injektion beobachtet. Dabei zeigte zu dieser Zeit die Gesamtcholesterinmenge bei den Hunden Nr. 1. und 2. keine Veränderung, während bei Hund Nr. 3. schon die Zunahme des Gesamtcholesterins konstatiert wurde. Die einmal verminderte Esterquote stieg in 2 ausgeheilten Fällen (Nr. 1. und 2.) unter einigen Schwankungen allmählich zum normalen Werte an, während sie bei einem—übrigens 14 Tage nach Injektion gestorbenen—Tiere (Nr. 3. 0.07 g Toluylendiamin pro Kilo verabreicht) nicht wieder anstieg. Einen vollkommenen Esterschwund haben wir nie beobachtet.

C. *Das Verhalten des Cholesterins und seiner Ester im Hundeblute bei gleichzeitiger Unterbindung der Ducti choledochus und cysticus.*

An 3 Hunden wurden die Ducti choledochus und cysticus gleichzeitig unterbunden und das Blut der Hunde wurde auf freies und Estercholesterin, gleichzeitig auf Serumbilirubin untersucht.

Die Resultate sind in Tabelle VI zusammengestellt.

In allen Fällen zeigte das Gesamtcholesterin eine erhebliche Vermehrung schon 5 bis 24 Stunden nach Operation und erreichte seine maximalen Werte nach 5 (Nr. 3.) resp. 13 Tagen (Nr. 1. und 2.). Dabei ging bis zum späteren Stadium der Versuche (7, 16 und 5 Tage) die Vermehrung der Estercholesterinmenge mit der des freien Cholesterins Hand in Hand, so dass die Esterquote keine Verminderung zeigte. Diese Beobachtung steht im Widerspruch zur monistischen Behauptung der Bürgerschen Schule, dass die Verminderung der Esterquote bei allen Fällen von Leber bezw. Gallenwegerkrankungen durch eine Störung der Fettresorption bezw. des intermediären Fettstoffwechsels infolge gehinderten Gallenabflusses zum Darm hervorgerufen wird.

Alle Hunde gingen 13, 27 bezw. 8 Tage nach der Operation ein. Kurz vor dem Tode wurde eine Verminderung der absoluten sowie prozentuellen Esterwerte gefunden. Diese Verminderung der Esterquote kann durch eine sekundäre Störung der Leberfunktion infolge Gallenstauung erklärt werden.

Zusammenfassung.

1. Der Gesamtcholesteringehalt im normalen Menschenvollblute variiert zwischen 0,128 und 0,163 g% ; dabei entspricht 37,5–47,9% des Gesamtcholesterins dem Estercholesterin.

2. Bei den Lebererkrankungen mit Parenchymschädigung wird eine absolute Verminderung des Estercholesterins bei normalem bezw. subnormalem Gesamtcholesterinwert im Blute konstatiert. Während die einmal verminderten Esterwerte unabhängig vom Stande des Gesamtcholesterins im Heilungsstadium wieder ansteigen, sinken sie beim Fortschreiten des Leberprozesses weiter ab.

3. Bei den Gallengangerkrankungen zeigen das Cholesterin und seine Ester im Blute verschiedenes Verhalten je nach dem Grad der Gallenwegsstörung und den Krankheitsphasen.

4. Beim hämolytischen Ikterus verhält sich der Cholesterinspiegel im Blute ganz normal.

TABELLE

Hund	Blutentnahme		Cholesterin im Gesamt-		
	Datum	Nach der Injektion (Stunden bzw. Tage)	Gesamt g%	Freies g%	Ester g%
Nr. 1.	5. IX (32.)	Vor der Injektion	0.115	0.062	0.053
♀	1^{h}pm „ „	5 Stden.	0.119	0.089	0.030
12,1 Kg	6^{h}pm „ „	10 „	0.155	0.099	0.056
	8^{h}am 6. IX	1 Tage	0.215	0.146	0.069
	„ 7.	2 „	0.192	0.134	0.058
	„ 8. „	3 „	0.148	0.101	0.047
	„ 9. „	4 „	0.135	0.090	0.045
	„ 10. „	5 „	0.151	0.080	0.071
	„ 11. „	6 „	0.166	0.105	0.061
	„ 12. „	7 „	0.173	0.102	0.071
Nr. 2.	19. IX (32.)	Vor der Injektion	0.120	0.062	0.058
♀	1^{h}pm „ „	5 Stden.	0.120	0.093	0.027
14.9 Kg	6^{h}pm „ „	10 „	0.131	0.093	0.038
	8^{h}am 20. IX	1 Tage	0.127	0.087	0.040
	„ 21. „	2 „	0.158	0.109	0.049
	„ 22. „	3 „	0.151	0.102	0.049
	„ 23. „	4 „	0.149	0.087	0.062
	„ 24. „	5 „	0.136	0.090	0.046
	„ 25. „	6 „	0.129	0.083	0.046
	„ 26. „	7 „	0.161	0.097	0.064
Nr. 3.	8. II. (33.)	Vor der Injektion	0.107	0.065	0.042
♀	1^{h}pm „ „	5 Stden.	0.132	0.111	0.021
8.0 Kg	6^{h}pm „ „	10 „	0.141	0.103	0.038
	8^{h}am 9. „	1 Tage	0.178	0.135	0.043
	„ 10. „	2 „	0.188	0.140	0.048
	„ 11. „	3 „	0.131	0.109	0.022
	„ 12. „	4 „	0.173	0.140	0.033
	„ 13. „	5 „	0.158	0.110	0.048
	„ 14. „	6 „	0.154	0.107	0.047
	„ 15. „	7 „	0.160	0.117	0.043
	„ 18. „	10 „	0.193	0.143	0.050
	„ 22. „	14 „	0.168	0.128	0.040

V.

blut	Serumblilirubin			
	Hijmans van den Bergh		Kumegawa mg%	Bemerkungen
Verestert %	direkte Reaktion	indirekte Reaktion mg%		
46.1	verzögert	spur	—	Um 8 Uhr am 5. IX (32.) wurde Toluylendiam (Merck) als 2.5%ige Lösung in der Dosis von 0.05 g. pro Kilo Körpergewicht subkutan injiziert.
25.2	unklar	unklar	unklar	
36.1	„	unmessbar (+)	unmessbar (+)	
32.1	prompt	5.00	18.10	
30.2	„	13.57	18.32	
31.8	„	7.14	9.64	
33.3	„	2.32	4.58	
47.0	„	1.43	2.89	
36.7	„	1.14	1.93	Am 12. IX (32.) abgetötet.
41.0	„	0.71	0.96	
48.3	(−)	(−)	(−)	Um 8 Uhr am 19 IX (32.) T. D. M. als 2.5%ige Lösung 0.05 gr. pro Kilo subkutan injiziert
22.5	unklar	unklar	—	
29.0	„	„	—	
31.5	prompt	1.07	—	
31.0	„	32.86	—	
32.5	„	10.00	19.29	
41.6	„	3.57	6.29	
33.8	„	1.00	1.06	
35.7	„	0.61	—	Am 26. IX (32.) abgetötet
39.8	„	0.61	—	
39.3	(−)	(−)	(−)	Um 8 Uhr am 8. II (33.) T. D. M. als 2.5%ige Lösung 0.07 gr. pro Kilo subkutan injiziert.
15.9	„	„	„	
27.0	unklar	unklar	unklar	
24.2	prompt	12.50	19.77	
25.5	„	14.29	26.04	
16.8	„	10.71	17.36	
19.1	„	8.57	14.46	
30.4	„	4.93	10.70	
30.5	„	4.82	8.68	
26.8	„	2.50	—	
25.4	„	1.86	—	Am 22. II (33.) gestorben.
23.8	„	1.14	—	

Tabelle

Hund	Blutentnahme		Cholesterin im Gesamt-		
	Datum (1932)	Nach der Operation (Stunden bzw. Tage)	Gesamt g%	freies g%	Ester g%
Nr. 1. ♀ 9.1 Kg	4. VII	Vor der Operation	0.104	0.058	0.046
	4^{h}pm „ „	5 Stden.	0.132	0.065	0.067
	9^{h}pm „ „	10 „	0.137	0.078	0.059
	11^{h}am 5. VII	1 Tage	0.143	0.073	0.070
	„ 6. „	2 „	0.139	0.073	0.066
	„ 8. „	4 „	0.149	0.083	0.066
	„ 11. „	7 „	0.143	0.079	0.064
	„ 14. „	10 „	0.145	0.105	0.040
	„ 17. „	13 „	0.154	0.115	0.039
Nr. 2. ♂ 7.5 Kg	27. VII	Vor der Operation	0.102	0.056	0.046
	4^{h}pm „ „	5 Stden.	0.104	0.061	0.043
	9^{h}pm „ „	10 „	0.110	0.066	0.044
	11^{h}am 28. VII	1 Tage	0.133	0.078	0.055
	„ 29. „	2 „	0.145	0.087	0.058
	„ 30. „	3 „	0.157	0.093	0.064
	„ 1. VIII	5 „	0.167	0.091	0.076
	„ 3. „	7 „	0.152	0.087	0.065
	„ 6. „	10 „	0.158	0.085	0.073
	„ 9. „	13 „	0.180	0.093	0.087
	„ 12. „	16 „	0.163	0.085	0.078
	„ 15. „	19 „	0.154	0.110	0.044
	„ 23. „	27 „	0.157	0.122	0.035
Nr. 3. ♀ 11.5 Kg	3. VIII	Vor der Operation	0.103	0.060	0.043
	4^{h}pm „ „	5 Stden.	0.119	0.076	0.043
	9^{h}pm „ „	10 „	0.143	0.096	0.047
	11^{h}am 4. „	1 Tage	0.150	0.100	0.050
	„ 5. „	2 „	0.138	0.086	0.052
	„ 6. „	3 „	0.151	0.087	0.064
	„ 8. „	5 „	0.161	0.090	0.071
	„ 10. „	7 „	0.158	0.119	0.039

VI.

blut	Serumbilirubin		Bemerkungen
	Hijmans van den Bergh		
Verestert %	direkte Reaktion	indirekte Reaktion mg%	
44.2	(−)	(−)	Um 11 Uhr am 4. VII endt die Operation von Unterbindung der Ducti choledochus und cysticus.
50.8	prompt	(−)	
43.1	„	unmessbar (⧺)	
49.0	„	1.93	
47.5	„	1.86	
44.3	„	4.57	
44.8	„	2.57	
27.6	„	6.43	Gegen 5 Uhr pm 17. VII gestorben
25.3	„	2.57	
45.1	(−)	(−)	Um 11 Uhr am 27. VII endet die Operation von Unterbindung der Ducti choledochus und cysticus.
41.3	unklar	unmessbar (+)	
40.0	prompt	unmessbar (+)	
41.4	„	unmessbar (⧺)	
40.0	„	0.64	
40.8	„	0.71	
45.5	„	1.70	
42.8	„	2.86	
46.2	„	2.86	
48.3	„	2.68	
47.9	„	0.57	
28.4	„	0.64	Gegen 7.5 Uhr pm 23. VII gestorben
22.3	„	1.07	
41.7	(−)	(−)	Um 11 Uhr am 3. VII endet die Operation von Unterbindung der Ducti cholodochus und cysticus.
36.1	prompt	unmessbar (⧺)	
32.9	„	0.36	
33.3	„	1.29	
37.7	„	3.21	
42.4	„	3.71	
44.1	„	2.89	Gegen 8 Uhr am 11. VIII gestorben
24.7	„	—	

5. Die Verminderung der Esterquote wird sowohl bei den Erkrankungen mit Hypercholesterinämie (essentielle Hypertonie, chronische Nephritis und Lipoidnephrose) als auch bei den mit Hypocholesterinämie (Anämie) nicht beobachtet.

6. Die Tagesschwankung des Cholesterins im normalen Hundeblute beträgt ca. 10% und die Esterquote zeigt fast keine nennenswerte Schwankung.

7. Bei mit Toluylendiamin vergifteten Hunden wird eine absolute Verminderung des Estercholesterins schon 5 Stunden nach der Giftinjektion beobachtet, und die Gesamtcholesterinmenge beginnt 5–10 Stunden nach der Injektion zuzunehmen und erreicht den maximalen Wert nach 1, 7 resp. 10 Tagen. Die einmal verminderte Esterquote steigt in ausgeheilten Fällen unter einigen Schwankungen allmählich zum normalen Werte an, während sie bei letalem Ausgang nicht wieder ansteigt.

8. Bei Hunden, deren Ducti choledochus und cysticus gleichzeitig unterbunden wurden, geht bis zum späteren Stadium der Versuche die Vermehrung der Estercholesterinmenge mit der des freien Cholesterins Hand in Hand, so dass die Esterquote keine Verminderung zeigt. Eine Verminderung der absoluten sowie prozentuellen Esterwerte wird kurz vor dem Tode gefunden.

9. Aus diesen Versuchsergebnissen wird geschlossen, dass in der Klinik einer ausführlichen Reihenuntersuchung des Cholesterins und seiner Ester eine wichtige Bedeutung für die Prognosesellung der Leber- bezw. Gallenwegerkrankungen zukommt.

Weitere Untersuchungen sind im hiesigen Laboratorium im Gang.

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ÜBER DIE OXYDATION DES ω-OXYMETHYL-FURFUROLS IM ORGANISMUS.

VON

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Die Oxydation der Hexosen wurde schon verschiedenartig untersucht und die Oxydationsprodukte analysiert. Die Ergebnisse können dahin zusammengefasst werden, dass die Oxydation nicht gleichmässig verläuft, sondern zu mannigfaltigen Oxydationsprodukten führt und in hohem Masse von den Versuchsbedingungen abhängig ist.

Bei Versuchen der Beeinflussung des Kohlehydratstoffwechsels steht das ω-Oxymethylfurfurol in einer besonderen Stellung, weil Steudel und Mitarbeiter (1924) wissen, dass zahlreiche Kohlehydrate, vor allem die Ketosen, bei geeigneter Zersetzung durch Säuren, mehr oder weniger grosse Mengen von Oxymethylfurfurol zu liefern imstande sind und dass seit Tollens in einer grossen Anzahl von Hexosen eine furanartige Struktur anzunehmen ist.

Karashima (1927) hat früher mitgeteilt, dass Tiere, denen frisch destilliertes Oxymethylfurfurol per os oder subcutan eingegeben worden ist, die Oxymethylbrenzschleimsäure zu mehr als 43% im Harn ausscheiden. Welches Organ bei diesem Vorgang die wesentliche Rolle spielt und wie weit er durch verschiedene Ernährungszustände beeinflusst wird, ist bisher gar nicht untersucht worden.

Um die erwähnten Fragen aufzuklären, habe ich einerseits einige Fütterungsversuche mit Oxymethylfurfurol an Hunden ausgeführt. Ich stellte meine Versuche zuerst bei Ernährung mit Eiweissfett und dann bei Hungerzustand an demselben, sonst gesunden Hunde an, mit dem ich auch vorher meine Versuche bei normaler gemischter Ernährung durchgeführt hatte. Andererseits wurden verschiedene isolierte Organe normaler, hungernder und mit Eiweiss-Fettdiät ernährter Hunde mit dem defibrinierten Blut desselben Tieres, gemischt mit Oxymethylfurfurol, durchströmt und danach aus dem durchströmten Blut die Oxymethylbrenzschleimsäure isoliert.

Die Menge der im Tierorganismus und in den isolierten Organen gebildeten Oxymethylbrenzschleimsäure dient dabei als Mass für die Oxydation des Oxymethylfurfurols, da die Säure sowohl im Tierorganismus als auch in den isolierten Organen sehr beständig ist und äusserst schwer angegriffen wird.

Aus meinen Versuchen geht hervor, dass das verabreichte Oxymethylfurfurol im normalen Hundeorganismus zu mehr als 95% oxydiert und als Oxymethylbrenzschleimsäure in dem Harn ausgeschieden wird. Dabei beobachtet man eine unverkennbare Herabsetzung der Oxydationsfähigkeit des Oxymethylfurfurols sowohl bei einseitiger Eiweiss-Fettdiät als auch bei Hunger. Weitere Durchblutungsversuche ergaben, dass der Hund in der Leber und zum kleineren Teil auch in dem Magen die Fähigkeit besitzt, das Oxymethylfurfurol zu Oxymethylbrenzschleimsäure zu oxydieren, aber in der Milz, Lunge und Niere ganz und gar nicht. Man bemerkt auch dabei, dass die Menge der durch Oxydation des Oxymethylfurfurols gebildeten Oxymethylbrenzschleimsäure in der Leber bei mit Eiweiss-Fettdiät ernährten oder hungernden Tieren immer geringer ist, als bei normalen mit kohlehydratreicher gemischter Kost gefütterten.

EXPERIMENTELLES.

1. *Fütterungsversuche:* 2 mit gemischter Nahrung gut genährten gesunden Hunden (K-G : A. 11,0 kg, B. 9,6 kg) wurden je 3 g Oxymethylfurfurol täglich per os gegeben. In 2 Tagen wurden 6 g Oxymethylfurfurol verabreicht. Der gesammelte Harn wurde abgedampft und in 90%igen Alkohol aufgenommen, der nach Abdampfen des Alkohols erhaltene Rückstand in wenig Wasser gelöst und unter Zusatz von Schwefelsäure mit Phosphorwolframsäure gefällt. Das Filtrat von der Phosphorwolframsäurefällung wurde mit Baryt zerlegt und aus dem alkalischen Filtrat das Barium mit Kohlensäure und dann mit Schwefelsäure ent-

fernt. Die salzfreie Lösung wurde nach dem Einengen mit Äther im Extraktionsapparat extrahiert. Aus dem Ätherextrakt schieden sich schon im Gang der Extraktion prachtvolle Krystallnadeln aus. Nach Abdampfen der ätherischen Mutterlauge erhielt man noch reichlich Krystalle. Die gereinigte Substanz schmolz bei 167° unter Gasentwicklung. In den Analysendaten zeigt diese Substanz eine gute Übereinstimmung mit Oxymethylbrenzschleimsäure.

Nach der Erholung des allgemeinen Zustandes wurden dieselben Hunde 1 Woche lang mit Eiweissfett ernährt und dann mit Oxymethylfurfurol in derselben Weise wie bei normaler Ernährung gefüttert.

Als nach mehreren Tagen ihre Ernährung und allgemeines Befinden sich ganz normal erholt hatten, stellte ich meinen Versuch bei Hunger, der sich über 7 Tage erstreckt, an denselben Hunden an. Die Verarbeitung auf Oxymethylbrenzschleimsäure aus dem Urin geschah in derselben Weise wie oben erwähnt.

Die Resultate sind in Tabelle I zusammengestellt.

Tabelle I.

Versuchstier.	Körpergewicht des Tieres.	Verfütterte Menge des Oxymethylfurfurols.	Gefundene Menge der Oxymethylbrenzschleimsäure.		Bemerkungen.
	in kg	in g	in g	in %	
Hund A	11,0	6,0	6,7	97,7	Gemischte Nahrung.
Hund B	9,6	„	6,5	95,0	„
Hund A	10,3	„	5,5	80,4	Eiweiss-Fettdiät.
Hund B	8,6	„	5,2	76,0	„
Hund A	8,5	„	6,5	95,0	Hunger.
Hund B	7,8	„	4,8	70,1	„

2. *Durchblutungsversuche:* Die Durchblutungsversuche wurden an Leber, Magen, Milz, Lunge und Niere der normalen, hungernden und mit Eiweiss-Fettdiät ernährten Hunde ausgeführt. Die Durchströmungsflüssigkeit bestand aus defibriniertem Hundeblut, vermischt mit 3-5 g frisch destilliertem Oxymethylfurfurol.

Um die gebildete Oxymethylbrenzschleimsäure krystallinisch zu erhalten, wurde das durchströmte Blut von einigen Versuchen gesammelt. Das gesammelte Blut wurde in Gegenwart von Schwefelsäure mit Phosphorwolframsäure enteiweisst, filtriert und dann mit Baryt behandelt. Aus dem alkalischen Filtrat wurde das Barium mit Kohlensäure und dann mit Schwefelsäure vollständig beseitigt. Die salzfreie Lösung wurde auf ein kleines Volumen eingeengt und im Flüssigkeitextraktor mit Äther extrahiert. Der Ätherextrakt wurde dann auf Oxymethylbrenzschleimsäure verarbeitet. Bei den Versuchen von Leberdurchblutung erhielt man reichliche Krystalle aus Ätherextrakt, die, aus heissem Wasser mit Tierkohle umkrystallisiert, bei 167°

TABELLE II.

Bei normaler Ernährung.

Versuchsnummer.	Körpergewicht des Hundes. kg	Organ.	Menge des Blutes. ccm	Menge des zugesetzten Oxymethylfurfurols. g	Durchblutungsdauer. Std	Gefundene Mengeder Oxymethylbrenzschleimsäure. g
1	10,0	Leber	300	3,0	3	3,60
2	12,5	„	400	4,0	„	
3	8,0	„	300	3,0	2	
4	10,5	„	400	4,0	„	
5	11,5	„	„	„	3	
6	8,0	Magen	300	5,0	3	0,12
7	9,5	„	„	3,0	„	
8	12,0	„	400	5,0	„	
6	10,5	Milz	300	3,0	2	(−)
10	11,0	„	„	„	„	
11	9,5	„	„	„	3	
12	12,5	Lunge	300	3,0	3	(−)
13	13,0	„	„	„	„	
14	8,0	„	„	„	2	
15	13,5	Niere	300	3,0	3	(−)
16	7,0	„	„	„	„	
17	12,0	„	„	„	„	

schmolzen. Dass es sich um Oxymethylbrenzschleimsäure handelte, wurde durch eine Analyse bestätigt.

0,0502 g:	0,9932 g	CO_2,	0,0200 g	H_2O,
$C_6H_6O_4$:	Ber.	C 50,69	H 4,26	
	Gef.	50,62	4,45	

Die Ergebnisse sind in folgender Tabelle zusammengestellt.

TABELLE III.
Leberdurchblutung.

Versuchsnummer.	Körpergewicht des Hundes. kg.	Gewicht der Leber. g.	Menge des Blutes. ccm.	Menge des zugesetzten Oxymethylfurfurols. g.	Durchblutungsdauer. Min.	Gefundene Menge der Oxymethylbrenzschleimsäure. g.	Bemerkungen.
18	12,7	450	350	3,0	80	1,3	Gemischte Nahrung.
19	11,8	500	„	„	„		
20	8,7	430	„	„	„	1,1	
21	13,0	550	„	„	75		
22	11,6	450	350	3,0	70	0,5	Eiweiss-Fettdiät.
23	9,7	360	„	„	80		
24	9,4	380	„	„	„	0,7	
25	10,0	420	„	„	75		
26	9,8	360	300	3,0	80	0,6	Hunger.
27	11,0	380	350	„	„		
28	8,0	„	300	„	„	0,5	
29	10,2	420	350	„	100		

ZUSAMMENFASSUNG.

Der Hund besitzt in der Leber und zum Teil im Magen die Fähigkeit, das Oxymethylfurfurol zu Oxymethylbrenzschleimsäure zu oxydieren.

Sowohl bei der einseitigen Eiweiss-Fettdiät als auch beim Hungern wird die Oxydationsfähigkeit des Oxymethylfurfurols im Hundeorganismus mehr oder weniger deutlich herabgesetzt.

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IS THE HYDROGEN GAS ELECTRODE NOT APPLICABLE TO THE DETERMINATION OF THE pH OF OXYGENATED BLOOD?

(Studies on the blood pH estimated by the glass electrode method. VI.)

By

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INTRODUCTION.

The hydrogen gas electrode is the standard method of pH determination. In using this method for the pH determination of blood, however, special precaution must be taken to exclude those errors due to loss of CO_2 (the CO_2-error), due to the existence of oxyhaemoglobin (the O_2-error), or due to the acid change caused by glycolysis. In practice, either Höber's or Hasselbalch-Michaelis' principle is generally employed. According to Höber's principle, hydrogen gas containing a certain amount of CO_2 is passed through the blood in the electrode vessel, and thus the blood is completely reduced without any loss of CO_2. Though the blood pH can be determined precisely and accurately by this method, the result is not that of the normal physiological condition but that of completely reduced blood. Using Hasselbalch-Michaelis' principle, a hydrogen gas bubble which has the same CO_2 pressure as that of the blood is introduced into the blood to exclude the CO_2-error. According to Michaelis(1914), this hydrogen gas bubble is prepared by introducing a small bubble of pure H_2 gas into the electrode. This method is more simple than Höber's as it can be applied directly to the blood under normal physiological conditions., i. e. in the oxygenated state, so that it was often used in classical studies. It is however doubted, whether or not the pH thus determined is the accurate pH of the oxygenated blood. In order to establish the accurate potential of the hydrogen gas electrode, the blood in the electrode or at least that touching the platinum electrode must be completely reduced, as was pointed out by Michaelis (1914), Konikoff (1913), Warburg (1922), etc. Therefore it does not appear that the pH value calculated from the potential thus established is the pH of the oxygenated blood. When the reduction of blood is not complete, the O_2-error ruins the accuracy of the measurements. Thus, Peters (1914) stated that it was impossible to determine the accurate pH of oxygenated blood until some other satisfactory electrode was found.

Since Parsons (1917) proved that whole blood has the same pH as that of its plasma, the pH of blood in the physiological, oxygenated condition is generally determined by separating the plasma and meauring its pH. For example, the plasma pH measured by the colorimetric method of Cullen (1922), Hastings and Sendroy (1924), or Hawkins (1923), and corrected to the value at the body temperature is usually regarded as the real pH of the blood.

One of us (Yoshimura, 1935, b) previously reported that the pH of oxygenated whole blood determined at a room temperature of 16°C by a hydrogen gas electrode (using Michaelis' principle) coincided approximately with that determined by means of a glass electrode. Thus he concluded that both the glass electrode and the hydrogen gas electrode can be applied to the pH determination of oxygenated whole blood. At the body temperature, however, the hydrogen gas electrode cannot be generally applied to the whole blood, as the acid change due to glycolysis progresses rapidly, while the potential of the electrode comes late to attain the equilibrium. Yoshimura (1935, b) also proved, using rabbit's blood, that the pH of its plasma separated at a room temperature and determined by means of a hydrogen gas electrode at 37° coincided with that of the whole blood measured by means of a glass electrode. Accordingly, he recommended that the plasma be applied to the hydrogen gas electrode measurement at body temperature.

The conclusions previously reported, however, include some weak points, as was suggested by Sendroy, Shedlovsky and Belcher (1936). The results should be closely reexamined to determine whether or not the oxygen error was completely excluded from the hydrogen gas electrode measurement of oxygenated whole blood; and whether the fact, that the pH of the plasma separated at a room temperature and measured at the body temperature coinciding with that of the whole blood, is applicable to other subjects than the rabbit. The present research was directed along these points.

METHODS.

The hydrogen gas electrode used in the present investigation, was Kurose's electrode of the syringe type (see Hiraki, 1932), which was based upon Michaelis' principle. The volume of hydrogen gas bubble introduced into it occupied 1/8–1/6 of its content. As the pH values of the plasma determined by the hydrogen gas electrode coincided within the scope of experimental error with that found by the use of the glass electrode measure-

TABLE I.

Comparison of hydrogen gas and glass electrode pH measurements on goat plasma at 15°C.

No. of experiment	P_{CO_2} of gas equilibrated	pH with glass electrode	pH with H_2 gas electrode	Difference (pH)
1	23 mm Hg	7.673	7.673	0.000
2	22	7.736	7.717	−0.019
3	43	7.404	7.398	−0.006
4	20	7.601	7.604	+0.003
5	16	7.589	7.610	+0.021
6	25	7.538	7.566	+0.028
7	26	7.534	7.548	+0.014
8	30	7.505	7.503	−0.002
9	16	7.637	7.634	−0.003
Mean				+0.004 ±0.003

ment (Table I), it is clear that this volume of gas does not cause any CO_2-error. Moreover this volume was found to be the best for obtaining the correct pH of oxygenated blood, when checked by means of the glass electrode. When the gas was introduced into oxygenated blood, however, its volume was chosen to be about 1/5 of that of the content. As a portion of it was eliminated by forming H_2O with oxygen coming from oxyhaemoglobin, its volume was reduced to about 1/6 of the content, when the equilibrium was attained.

In order to correct the E. M. F. of the hydrogen gas electrode against a decrease of the partial pressure of hydrogen gas caused by CO_2 coming from the blood, the blood used in the measurement was previously equilibrated at the temperature of the measurement (15°) with a gas of a certain CO_2 pressure (16–43 mm Hg), which was measured with a Haldane apparatus. The correction was calculated from this partial pressure of CO_2.

The glass electrode was the type of capillary tubing previously reported (Yoshimura, 1935, a) with the modification that a *U* tubing of MacInnes glass ca. 0.3 mm thick and 6 cm long replaced the electrode membrane. Its capacity was ca. 0.7 cc. With such an electrode, it was often found that the potential attained an equilibrium very slowly, and that its slope of the potential-pH line was slower than the theoretical value. Therefore, those electrodes with which the equilibrium of the potential could be attained within 5 minutes and the slope of the potential-pH line coincided with the theoretical value were selected.

In order to compare the pH determined by the hydrogen gas electrode method with that found by the glass electrode method, both sorts were always checked with a phosphate buffer of a known pH (pH′) before beginning the experiments of the day. This pH was previously determined by means of the hydrogen gas electrode method. 0.01 N HCl, the pH of which was assumed to be 2.035 at 15°C and 2.036 at 37°C, was adopted as the standard according to Katsu (1927). Now the potential of this buffer measured with any of the two sorts of electrodes being E', and that of the blood with the same electrode E, the pH of the blood is found by

$$\mathrm{pH} = \mathrm{pH}' + \frac{E - E'}{RT/F}, \qquad (1)$$

where R is the gas constant expressed in a corresponding unit. The pH thus obtained with the hydrogen gas electrode and that with the glass electrode are to be compared. By using this procedure, an accurate comparison is possible, disregarding the diurnal change in the calomel electrode potential, in the atomospheric pressure, and in the asymmetry potential of the glass electrode, etc.

To secure the accuracy of the data, the same sample was measured repeatedly with one or two glass electrodes, and with 4–11 hydrogen gas electrodes and the data observed were respectively averaged.

It was found by preliminary experiments that oxalated goat blood kept its pH constant over 6 hours at 15°C, owing to the very slow progression of its glycolysis. Therefore this blood was generally adopted for the experiments in which the applicablility of the hydrogen gas electrode method to the oxygenated blood sample was to be examined. In these experiments, a long time was allowed in order to attain the potential equilibrium. In other experiments, the oxalated blood samples of various animals were also used. These blood samples were usually kept at 15°C or below, except when the temperature is mentioned in the description. On the preparation of the oxalated blood, see our previous report (Yoshimura, b).

To avoid a loss of CO_2 from the blood, it was always treated under a thick layer (ca. 4 cm) of liquid paraffin except when it was necessary to keep the blood over ca. 2 hours. In the latter case, solid paraffin was superposed on the liquid paraffin layer, or the blood was kept in a closed vessel with a stopcock attached.

On the measurement of the glass electrode potential by means of an electron tube electrometer.

The potential of the glass electrode was measured with an electron tube electrometer which was constructed with a space charge grid tube Mazda *UX* 54 (Hamada, 1933). The circuit design was that of the ordinary simple type (cf. Fig. 2 of Müller and Dürichen). When this electrometer was used as a null instrument with a galvanometer of a sensibility of ca. 2×10^{-8} A., it could accurately measure the potential up to 0.1 m. V. In spite of its high input resistance (ca. $10^{15}\Omega$), an error due to a grid current which amounted to several tenths millivolts was found, when a glass electrode with a resistance of ca. $10^{9}\Omega$ was measured. This error was easily eliminated by the following procedure suggested by Kahler, De Eds, Rosenthal and Voegtlin (1930): Let the real potential of the glass electrode be E, its internal resistance R, and the grid current I_g, the sign of which being positive when the grid current flows from grid to filament in the interior of the tube. Then the positive pole of the glass electrode being connected with the potentiometer, and the negative pole to the control grid, the potential E_+ which is given by the potentiometer reading is expressed by the following formula:

$$E_+=E+I_gR \qquad (2).$$

Again, the connection of the electrode being reversed, the potential E_-, given by the reading, is expressed by

$$E_-=E-I_gR \qquad (3).$$

On adding (2) to (3), the real potential is obtained by

$$E=\frac{E_++E_-}{2} \qquad (4).$$

By subtracting (3) from (2), we have the grid current,

$$I_g=\frac{E_+-E_-}{2R} \qquad (5).$$

Thus the real potential of the electrode can be calculated simply from the equation (4), the error due to the grid current being eliminated. The grid current is also calculated from the equation (5) without any special measurement, usually done by inserting in the grid circuit a known high resistance having no potential of its own.

The fact theoretically derived was verified by the experiment the results of which are given in Table II. As is seen in it, the real potential calculated from the equation (4) coincides well with that determined with a Lindemann electrometer, while the potential actually read on the potentiometer varies as the grid potential changes. The grid current calculated from the equation (5) also coincides within the scope of experimental error with that determined by the use of the vacuum tube resistor of $1.3\times10^9\Omega$ (Tokyo Elect. Co.).

TABLE II.

Grid bias	Potential determined with the tube electrometer			Grid current	
	E_+	E_-	E	Calculated	Observed
−1.9 V	196.6 mV	194.8 mV	195.7 mV	$+3.6\times10^{-13}$ A	$+4.6\times10^{-13}$ A
−2.4	195.6	195.8	„	$-0.4\times$ „	$-0.1\times$ „
−2.9	195.5	195.9	„	$-0.8\times$ „	$-0.8\times$ „
−3.9	195.4	196.0	„	$-1.2\times$ „	$-1.3\times$ „

Electrode measured: Glass electrode of $2.5\times10^9\Omega$ resistance of which the potential determined with a Lindemann electrometer was 195.7 mV.

Conditions at which the electrometer was operated: Filament current 0.1 A, Plate voltage 7.2 V, Space charge grid potential 5.2 V.

In order to measure the pH by means of a glass electrode, however, such special precautions for eliminating the error due to the grid current is unnecessary, for the error can be eliminated in calculating the pH by the equation (1) in so far as the grid current is kept constant within the scope of experimental error throughout the experiment. When the pH values of the same buffer solution or blood measured by means of this electron tube electrometer and by Lindemann's electrometer were compared with each other, it was found that they coincided within ca. 0.01 pH, which corresponds approximately to the reproducibility of the potential of the glass electrode.

Experimental Results and Discussion.

A) Is the Hydrogen Gas Electrode not Applicable to the pH Determination on an Oxygenated Blood at a room Temperature?

To answer the question, it is best to compare the pH of the same oxygenated blood determined by means of a hydrogen gas electrode and of a glass electrode. Preliminary to the experiment with the oxygenated blood, however, it is necessary to confirm the applicability of the glass electrode to the pH determination of whole blood, as pointed out by Sendroy, Shedlovsky and Belcher (1936). Although the reliability of the glass electrode measurement of the plasma pH was proved by several investigators, e. g. Stadie, O'brien and Laug (1931), Haugaard (1934), Yoshimura (1935) etc, the only approximate proof of it for the whole blood was offered by Sendroy, Shedlovsky and Belcher (1936).

To check the results of the latter authors, a comparison between the glass electrode measurement and the hydrogen gas electrode measurement was made at 15°C on the reduced whole blood of goat and also on its reduced sample haemolysed by 1% saponin. Reduction of the blood was accomplished by passing pure hydrogen gas over the blood in a saturator, and then

Table III.

Comparison of hydrogen gas and glass electrode pH measurements on reduced whole blood of goats at 15°C.

Sample		pH with glass electrode	pH with H_2 gas electrode	Difference	Mean
Whole blood	1	7.330	7.336	+0.006 pH	+0.002 pH
	2	7.510	7.504	−0.006	
	3	7.495	7.469	−0.026	
	4	7.317	7.349	+0.032	
Haemolysed blood	1	7.383	7.389	+0.006	+0.008
	2	7.442	7.461	+0.019	
	3	7.435	7.433	−0.002	

it was equilibrated with a hydrogen gas containing CO_2 of the physiological tension in a tonometer. Results are given in Table III. It can be seen that the pH values of the reduced blood determined by means of both sorts of electrodes coincide with each other almost within the scope of experimental error. Thus we can determine the pH value of whole blood and also of haemolysed blood by means of our glass electrode.

Then similar comparisons were carried out at 15°C on the oxygenated whole blood of goats and also on the oxygenated sample haemolysed by 1% saponin. Results are given in Tables IV and V. In the second column of the tables, the partial pressure of oxygen equilibrated with the blood is given which is sufficiently high to make the blood fully oxygenated. As is seen in Table IV, the average pH difference between the values determined by both sorts of electrodes is less than its probable error. Therefore it is confirmed that the hydrogen gas electrode can be applied to the pH determination of oxygenated whole blood at room temperature. Table V shows, however, that the

TABLE IV.

Comparison of hydrogen gas and glass electrode pH measurements on oxygenated whole blood of goats (15°).

No. of experiment	Po_2 of gas equilibrated	pH with glass eletrode	pH with H_2 gas electrode	Difference
1	150 mm Hg	7.663	7.669	+0.006 pH
2	150	7.704	7.697	−0.007
3	153	7.737	7.730	−0.007
4	150	7.343	7.321	−0.022
5	152	7.581	7.600	+0.019
6	154	7.602	7.602	0.000
7	130	7.504	7.539	+0.035
8	125	7.299	7.279	−0.020
9	154	7.389	7.414	+0.025
10	153	7.474	7.467	−0.007
Mean				+0.002 ±0.004

TABLE V.

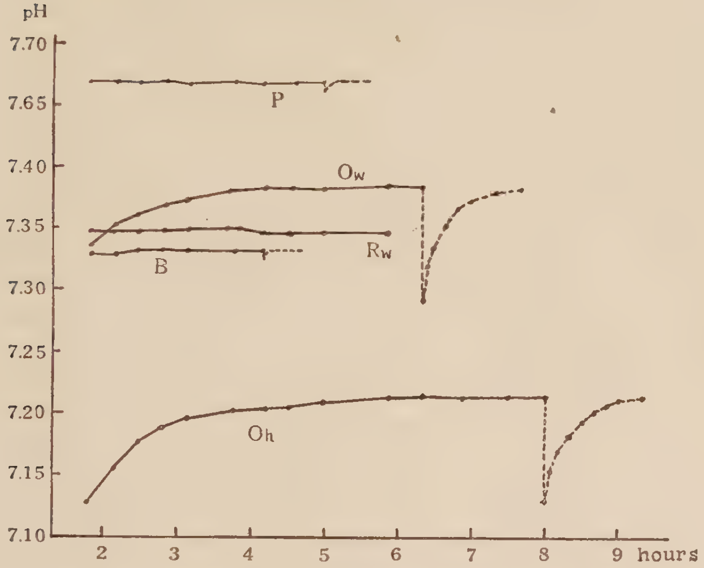
Comparison of hydrogen gas and glass electrode pH measurements on oxygenated goat blood haemolysed with 1% saponin.

No. of experiment	Po_2 of gas equilibraated	pH with glass electrode	pH with H_2 gas electrode	Difference (pH)
1	150 mm Hg	7.522	7.560	+0.038
2	134	7.434	7.485	+0.051
3	125	7.189	7.229	+0.040
4	127	7.329	7.412	+0.083
5	154	7.213	7.265	+0.052
6	153	7.237	7.316	+0.079
Mean				+0.057 ±0.006

pH values of the oxygenated haemolysed blood determined with the hydrogen gas electrode is always higher by 0.04–0.08 pH than that estimated with the glass electrode; and the mean difference (0.06 pH) between these two pH values is ten times greater than its probable error (±0.006 pH). Thus there is no doubt but that this difference really exists. Similar results were obtained on the oxygenated whole blood of a horse (partly reported in the previous paper, Yoshimura, b) and also on a haemolysed sample of the same blood.

Now, in order to answer the question why the two deternations are in discord on the haemolysed blood while they are in accord on the whole blood, the following experiments were carried out: (1) The courses of the hydrogen gas electrode were followed after the electrode being shaken for 1.5 hours in various solutions. The figure shows the results. The potential is taken on its ordinate in the pH unit, while the time after hydrogen gas bubble being introduced into the electrode is taken on its abscissa. While the potential attained an equilibrium even at the beginning in a phosphate buffer solution, plasma or reduced blood, the equilibrium was attained barely after ca. 4 hours in oxygenated whole blood, and after ca. 6 hours in oxygenated haemolysed blood. The delay of the potential equilibra-

Fig.
Courses of potential (in pH unit) of hydrogen gas electrode with the lapse of time in various solutions.



tion in the oxygenated blood samples was due to the prolonged reduction of an abundant quantity of oxygen on the surface of the platinum electrode. After the potential attained the equilibrium in the oxygenated blood, the electrode was shaken 2–3 times to stir the solution. Then it was observed that the potential fell immediately to a markedly low value and thereafter it rose gradually to attain the initial equilibrated potential. This is shown by the dotted line in the figure. In these cases, the electrode vessel was not dipped directly in a saturated KCl solution, but it was immersed in blood of the same source, which was connected with the saturated calomel electrode by a saturated KCl agar bridge. Therefore this potential fall was by no means due to the contamination of the blood with the saturated KCl solution. A similar potential fall caused by shaking was also seen in the case of the buffer solution and the plasma, but its extent was far less than that of the oxygenated blood. Therefore this marked potential fall found with the latter was thought to be due to liberation of oxygen on the surface of the platinum electrode. This indicates that, the portion of blood directly touching the platinum electrode being almost completely reduced, the potential of the hydrogen gas electrode applied to an oxygenated blood attains an equilibrium, even when a large amount of oxyhaemoglobin remains in the main portion of the blood. (2) The question arises as to what extent the oxygenated samples, thus brought into a potential equilibrium, were reduced. To answer the question, the gaseous contents of the oxygenated blood were once analysed by a Van Slyke and Neill apparatus before it was mounted in the hydrogen gas electrode, and once again after it was treated similarly to the usual hydrogen gas electrode measurement but in this case the dipping in a saturated KCl solution was ommited in order to prevent the blood from contamination. The whole and the haemolysed blood were examined at the same time. The results were that only a partial reduction had occurred in the electrode vessel, i. e. 22 – 34% reduction in the oxygenated whole blood, and 33 – 52% reduction in the haemolysed one, the latter always exceeding the former. The CO_2 content of the blood was found to be approximately unchanged by the equilibration. This fact ensures that any appreciable CO_2-error did not occur in the hydrogen gas electrode measurement. An example is given in Table VI. (3) It is asked if such a degree of reduction of the oxygenated blood samples might affect their pH. To answer the question, the

TABLE VI.

Comparison of gaseous contents of oxygenated goat blood before and after the treatment with the hydrogen gas electrode.

Sample	Gas	Before	After	% reduction
Whole blood	CO_2	49.1%	48.5%	22%
	O_2	12.7	9.9	
	% oxygenation	100		
Haemolysed blood	CO_2	51.7	51.5	33
	O_2	12.3	8.2	
	% oxygenation	98		

pH being determined by the glass electrode method, comparisons of the pH values of an oxygenated samples before and after reduction in the hydrogen gas electrode were made similarly to the above experiment of gas analysis. Results tell us that the pH of the whole blood thus reduced was not changed, while that of the haemolysed blood was increased on an average by 0.03 pH (see Table VII). This difference of the effect of the reduction upon the pH of whole blood and haemolysed blood may be explained by the following three reasons. (i) The haemolysed blood is reduced in the electrode to a higher degree than the whole blood as mentioned above. (ii) As was pointed out by Van Slyke, Hastings and Neill (1922), the amount of alkali liberated from oxyhaemoglobin by a certain degree of reduction is larger in the case of haemolysed blood than in the case of whole blood. (iii) The Donnan equilibrium which causes a buffering power against the pH shift due to the reduction of oxyhaemoglobin is lost in the haemolysed sample. These are probably the reasons why the pH of the haemolysed blood was increased by the partial reduction in the hydrogen gas electrode, while that of the whole blood was not changed, probably owing to its high buffer capacity.

TABLE VII.

Comparison of pH of oxygenated goat blood before and after the treatment with the hydrogen gas electrode.

Sample	No. of experiment	Before	After	Difference	Mean
Whole blood	1	7.704 pH	7.698 pH	−0.006 pH	−0.001 pH
	2	7.389	7.394	+0.005	
	3	7.766	7.764	−0.002	
Haemylosed blood	1	7.565	7.590	+0.025	+0.032
	2	7.213	7.265	+0.052	
	3	7.639	7.657	+0.018	

From these facts, we can explain why the hydrogen gas electrode gave a correct pH for oxygenated whole blood, but a little higher one for oxygenated haemolysed sample. In measuring the pH of oxygenated whole blood with the hydrogen gas electrode, what was really touching the platinum electrode was the plasma, as the electrode vessel was kept upright and the corpuscles settled down from the layer immersing the electrode. As the pH of the plasma was not changed by a complete reduction, the pH of the plasma touching the platinum electrode was the same as that of the main portion of the whole blood in the electrode which was proved no different from the pH of the initial oxygenated whole blood. Thus we see that the potential of the hydrogen gas electrode gave the real pH of the initial oxygenated whole blood. In the case of haemolysed blood, however, not only the pH of the blood in the electrode as a whole was shifted to a little higher pH owing to the partial reduction by the hydrogen gas as mentioned above, but also the pH of the portion directly touching the platinum electrode was further increased by its almost complete reduction. These are probably the reasons why the hydrogen gas electrode gave a decisively higher pH value than the glass electrode.

In short, the hydrogen gas electrode can be applied to the pH determination of the oxygenated whole blood at a temperature of about 15°C. The pH of the oxygenated haemolysed blood determined by the hydrogen gas electrode method is, however, higher by 0.06 pH on an average than the correct pH owing to the reduction of oxyhaemoglobin. Thus the datum is only reliable to 0.1 pH. Refering to the present results, data obtained in classical investigations on the acid-base equilibrium of blood may be regarded as approximately correct.

The hydrogen gas electrode method is, however, by no means the best for determining the pH of oxygenated blood, as its experimental error is rather serious, probably owing to the CO_2-error and the O_2-error which are not completely eliminated. For example, ±0.013 pH was obtained as the probable error of a single observation calculated from ten repeated determinations of one and the same oxygenated whole blood, while ±0.004 pH was the probable error for reduced whole blood.

B) Is the Hydrogen Gas Electrode Applicable to the pH Determination of Oxygenated Blood at Body Temperature?

To estimate the blood pH at body temperature by means of the hydrogen gas electrode, one of the present authors (Yoshimura, b) previously recommended that it be done by measuring the plasma separated at room temperature, as glycolysis cannot be avoided in the whole blood. To apply this generally to any sort of animal blood, however, it is necessary to confirm that the temperature coefficient of the pH of the whole blood is the same as that of its plasma.

Among classical investigations, Michaelis and Davidoff (1912) found that the pH of human blood measured by means of hydrogen gas electrode has the same temperature coefficient as that of its plasma, their pH decreasing on a temperature rise. A similar result was ascertained colorimetrically by Martin and Leppar (1926) on diluted human blood. On the contrary, Hou (1926) found electrometrically that the pH of the whole blood of horse decreases 0.09-0.16 pH more intensively than that of its separated plasma when the temperature changes from 18° to 37°.

While in the above experiments the total CO_2 content was kept constant on changing the temperature, Hasselbalch (1917) found, on keeping the partial pressure of CO_2 constant, that the temperature coefficient of the pH of a whole blood and its separated plasma differed.

In these investigations, however, they did not pay due attention to the influence of glycolysis taking place in the sample after collection. According to present knowledge, this may not be neglisible at all. It sometimes causes a marked change of the pH of the sample. Therefore the problem was re-examined during the present research, the glass electrode method being applied to avoid any influence of glycolysis. For this purpose, it is best to compare the temperature coefficient of whole blood with that of its separated plasma, the pH measurement being carried out on each of both samples at various temperatures. In order to simplify the procedure, however, the question was answered in the present research by comparing the pH of the plasma separated at various room temperatures but measured at 37°C with that of its original whole blood measured at the same temperature. If the temperature coefficients of both samples are not the same, their pH will differ, and the difference will become larger as the temperature of plasma separation falls.

At first the effect of the separation of the plasma at 10°–15°C was examined, of which the results are given in Table VIII, 1–8. The blood was drawn from the cubital vein of human subjects (1), the jugular vein of goats (4) and of a horse (5), and from the carotid artery or by heart puncture of cats (2), of dogs (3), of rabbits (6), of cocks (7) and of toads (8). Its pH (given in "a" of the second column in each table) was determined immediately after shedding at 37°C. Another portion was centrifuged after being cooled down to a room temperature of 10°–15°, and its plasma was determined on its pH at 37°C (4 th column in the table). Then the remaining content of the centrifuge tube was stirred with a glass rod, and the pH of the whole blood thus restored was again measured at the same temperature ("b" of the second column). During two measurements, the pH of the whole blood coincided within ca. 0.01 pH. Thus the mean value of the two tests was taken as the pH of the whole blood. The pH of the plasma was always higher than that of the whole blood, the average differences being as follows: 0.08 for human blood, 0.10 for cat blood, 0.10 for dog blood, 0.06 for goat blood, 0.06 for horse blood, 0.02 for rabbit blood, 0.11 for cock blood and 0.10 for toad blood.

Next, similar experiments were carried out on the blood of cats and rabbits, the plasma being separated at 20° 25°C. The pH difference between the whole blood and its plasma was 0.05 pH in the case of the cat (Table VIII, 9), i. e. about half of the pH difference in the above case, while a pH difference of 0.001 which lies within the scope of experimental error was

TABLE VIII.

Comparison of the pH values of whole blood and plasma at 37°C.

1) Human blood and its plasma separated at 10°–15°C.

No. of experiment		Whole blood		Plasma	Difference
		Observed	Mean		
1	a	7.244 pH	7.245 pH	7.328 pH	+0.083 pH
	b	7.246			
2	a	7.306	7.302	7.382	+0.080
	b	7.297			
3	a	7.279	7.274	7.351	+0.077
	b	7.269			
4	a	7.255	7.249	7.333	+0.084
	b	7.242			
5	a	7.328	7.325	7.406	+0.081
	b	7.322			
Mean					+0.081

TABLE VIII.

2) Cat blood and its plasma separated at 10°–15°C.

No. of experiment		Whole blood		Plasma	Difference
		Observed	Mean		
1	a	7.307 pH	7.302 pH	7.392 pH	+0.090 pH
	b	7.296			
2	a	7.273	7.268	7.383	+0.115
	b	7.262			
3	a	7.239	7.226	7.327	+0.101
	b	7.213			
4	a	7.231	7.222	7.309	+0.087
	b	7.213			
5	a	7.206	7.205	7.305	+0.100
	b	7.203			
6	a	7.338	7.338	7.424	+0.086
	b	7.338			
Mean					+0.097

TABLE VIII.

3) Dog blood and its plasma separated at 10°–15°C.

No. of experiment		Whole blood		Plasma	Difference
		Observed	Mean		
1	a	7.211 pH	7.208 pH	7.307 pH	+0.099 pH
	b	7.205			
2	a	7.169	7.168	7.260	+0.092
	b	7.166			
3	a	7.179	7.177	7.273	+0.096
	b	7.175			
4	a	7.156	7.147	7.244	+0.097
	b	7.138			
Mean					+0.096

TABLE VIII.

4) Goat blood and its plasma separated at 10°–15°C.

No. of experiment		Whole blood		Plasma	Difference
		Observed	Mean		
1	a	7.302 pH	7.303 pH	7.365 pH	+0.062 pH
	b	7.304			
2	a	7.304	7.300	7.360	+0.060
	b	7.296			
3	a	7.377	7.376	7.436	+0.060
	b	7.374			
4	a	7.296	7.294	7.357	+0.063
	b	7.292			
5	a	7.327	7.326	7.405	+0.080
	b	7.325			
6	a	7·361	7.364	7.437	+0.073
	b	7.367			
7	a	7.375	7.348	7.410	+0.062
	b	7.338			
8	a	7.356	7.356	7.410	+0.054
	b	7.356			
Mean					+0.064

TABLE VIII.

5) Horse blood and its plasma separated at 10°–15°C.

No. of experiment		Whole blood		Plasma	Difference
		Oberved	Mean		
1	a	—	7.354 pH	7.390 pH	+0.046 pH
	b	7.354 pH			
2	a	7.339	7.344	7.396	+0.052
	b	7.349			
3	a	7.325	7.323	7.385	+0.062
	b	7.321			
4	a	7.339	7.339	7.386	+0.047
	b	7.339			
5	a	7.346	7.344	7.403	+0.059
	b	7.341			
6	a	7.268	7.271	7.350	+0.079
	b	7.273			
7	a	7.346	7.336	7.394	+0.058
	b	7.326			
8	a	7.315	7.313	7.365	+0.052
	b	7.312			
Mean					+0.057

TABLE VIII.

6) Rabbit blood and its plasma separated at 10°–15°C.

No. of experiment		Whole blood		Plasma	Difference
		Observed	Mean		
1	a	7.244 pH	7.243 pH	7.260 pH	+0.017 pH
	b	7.242			
2	a	7.278	7.280	7.301	+0.021
	b	7.281			
3	a	7.283	7.273	7.304	+0.031
	b	7.263			
Mean					+0.023

TABLE VIII.

7) Cock blood and its plasma separated at 10°–15°C.

No. of experiment		Whole blood		Plasma	Difference
		Observed	Mean		
1	a	7.427 pH	7.424 pH	7.536 pH	+0.112 pH
	b	7.421			
2	a	7.383	7.381	7.504	+0.123
	b	7.379			
3	a	7.392	7.385	7.499	+0.114
	b	7.379			
4	a	7.396	7.400	7.505	+0.105
	b	7.403			
Mean					+0.114

TABLE VIII.

8) Toad blood and its plasma separated at 10°–15°C.

No. of experiment		Whole blood		Plasma	Difference
		Observed	Mean		
1	a	7.117 pH	7.123 pH	7.240 pH	+0.097 pH
	b	7.132			
2	a	7.405	7.386	7.484	+0.098
	b	7.367			
3	a	7.106	7.093	7.203	+0.110
	b	7.084			
4	a	7.439	7.429	7.515	+0.086
	b	7.419			
Mean					+0.098

TABLE VIII.

9) Cat blood and its plasma separated at 20°–25°C.

No. of experiment		Whole blood		Plasma	Difference
		Observed	Mean		
1	a	7.310 pH	7.292 pH	7.336 pH	+0.044 pH
	b	7.273			
2	a	7.314	7.294	7.361	+0.067
	b	7.273			
3	a	7.283	7.274	7.323	+0.049
	b	7.265			
Mean					+0.053

observed in the case of the rabbit (Table VIII, 10). The latter fact coincides with the result found in our previous report.

Finally, the plasma separated at ca. 37°C was studied. For this purpose, a special centrifuge tube was used. This tube was constructed in the following manner: Inside a metal tube was

TABLE VIII.

10) Rabbit blood and its plasma separated at 20°–25°C.

No. of experiment		Whole blood		Plasma	Difference
		Observed	Mean		
1	a	7.357 pH	7.353 pH	7.357 pH	+0.004 pH
	b	7.348			
2	a	7.369	7.357	7.354	−0.003
	b	7.344			
4	a	7.357	7.357	7.344	−0.013
	b	7.357			
5	a	7.356	7.351	7.367	+0.016
	b	7.346			
Mean					+0.001

TABLE VIII.

11) Goat blood and its plasma separated at 30°–50°C.

No. of experiment		Whole blood		Plasma	Difference
		Observed	Mean		
1	a	7.369 pH	7.365 pH	7.380 pH	+0.015 pH
	b	7.361			
2	a	7.366	7.355	7.362	+0.007
	b	7.344			
3	a	7.366	7.362	7.362	0.000
	b	7.357			
4	a	7.327	7.329	7.333	+0.004
	b	7.331			
5	a	7.361	7.364	7.388	+0.024
	b	7.367			
Mean					+0.010

placed a tube of glass. This was in turn lined with asbestos, and then was added another glass tube, liquid paraffin, and still another glass tube.

This six-walled tube being previously warmed at ca. 50°C, the blood was put into it under liquid paraffin, and it was immediately centrifuged for ca. 15 minutes in a room of ca. 30°C. The pH of the whole blood of goats and that of its plasma thus separated between 30°–50°, both being estimated at 37°C, are given in Table VIII, 11. The pH of the whole blood coincides approximately with that of its plasma (within 0.01 pH on an average), though the former was in most cases lower than the latter. The latter fact was probably due to the fact that the temperature of the plasma separation was lower than 37° and also to an acid change caused by glycolysis. A similar experiment was carried out at 15°, and it was ascertained that the whole blood and its separated plasma has the same pH (Table VIII, 12). In this experiment, the pH of the whole blood was measured once after determination of the plasma pH.

TABLE VIII.

12) Goat blood pH at 15°, the plasma being separated at ca. 15°C.

No. of experiment	Whole blood	Plasma	Difference
1	7.448 pH	7.457 pH	+0.009 pH
2	7.427	7.441	+0.014
3	7.436	7.434	−0.002
4	7.602	7.593	−0.009
5	7.596	7.576	−0.020
6	7.598	7.596	−0.002
7	7.595	7.607	+0.012
Mean			+0.0003

Thus it was found that the pH of the plasma separated at a low temperature is higher than that of its whole blood when measured at 37°; and the pH difference increases as temperature of the plasma separation decreases, while they coincide with each other when the temperature of the pH determination is the same as that of the plasma separation. These facts indicate that the temperature coefficient (in absolute value) of the whole blood is greater than that of its separated plasma, the pH of both the whole blood and its plasma decreasing with temperature rises, when their total CO_2 content is kept constant.

From this result it might be maintained that when the pH of a blood is to be determined by the hydrogen gas electrode method at body temperature, the plasma separated at the same temperature should be applied to the measurement with the exception of rabbit blood, of which the plasma separated at 20°–25°C gives the correct pH. Actually, in the above experiment on goat blood, an approximately correct pH was obtained on the plasma thus separated at ca. 37°C. But this should be restricted to goat blood in which the progress of glycolysis is slow even at the body temperature. The latter fact can be seen in Table IX, where is sighted the general aspect of the magnitude of the acid change due to glycolysis in oxalated whole blood of various animals. The figures in the table are decreases of blood pH during the lapse of 15 minutes or so after shedding which were measured by the glass electrode method at 37°C. As is seen in it, the progress of glycolysis is, however, rather

TABLE IX.

Acid change occurred during the lapse of ca. 15 minutes after shedding in oxalated whole blood.

Animal	Nos. of experiment	Acid change in pH unit	
		Extent of the change	Mean
Human	4	0.006–0.023	0.016
Cat	7	0.015–0.065	0.039
Dog	6	0.010–0.036	0.023
Rabbit	11	0 — 0.033	0.018
Horse	5	0 — 0.016	0.008
Goat	10	0 — 0.020	0.008
Cock	7	0 — 0.015	0.006
Toad	6	0 — 0.023	0.014

rapid in cat blood, dog blood, etc. Therefore, on these sorts of blood, the accurate pH can not be obtained by applying the plasma separated at the body temperature to the measurement, as an error due to the acid change may occur during the plasma separation.

From these results, it is concluded that the hydrogen gas electrode is not generally applicable to the pH determination of oxygenated whole blood at body temperature, the glass electode measurement being the only accurate one for the purpose. As mentioned in the introduction, plasma separated at room temperature is now generally used for pH determination in the methods of Cullen, Hastings and Sendroy, Hawkins, etc. However, the pH values of human blood hitherto determined by these methods and generally believed as valid seem to be somewhat higher than the real pH.

Now we will discuss the reason why the temperature coefficients of the whole blood and its plasma differ from each other. As the blood is a buffer solution consisting of several acid buffer systems, such as protein, bicarbonate and phosphate systems, the temperature coefficient of its pH depends ultimately upon those of the dissociation constants of the acids and the relative proportion of their buffer values. As is well known, the dissociation constants of these weak acids increase when the temperature rises, that is, the heat of dissociation is negative. This is the cause of the decrease of the blood pH accompanied by the temperature rise. According to van't Hoff's isochore, the temperature coefficient (in absolute value) of the dissociation constant is exponentially proportional to the absolute magnitude of the heat of dissociation. It has been well known since Meyerhof's calorimetric experiment that protein has the largest or enormous heat of dissociation (in absolute value) among the acids, that is, the temperature coefficient of the dissociation constant of protein is the largest. This is the reason why the temperature coefficient of the pH of the plasma is less than that of the whole blood, the whole blood being far richer than the the plasma in protein (haemoglobin), and the buffering power of the whole blood being mainly contributed by protein.

The fact that the difference between the pH change of the whole blood and that of its separated plasma, due to temperature change, varies according to the species of animal, is thought to be mainly due to the variety of the heat of acid dissociation and the quantity, i. e. the buffering power, of protein it contains.

RESUME.

1. On goat and horse blood, it was proved that the hydrogen gas electrode on Michaelis' principle can be applied to the pH determination of the oxygenated whole blood at a room temperature (15°C) at which the speed of glycolysis is slow. In this case the material directly touching the platinum electrode is mainly its plasma the pH of which does not change on reduction. The pH of the oxygenated haemolysed blood measured by means of the hydrogen gas electrode is, however, higher (by 0.06 pH on an average in the case of goat blood) than its real pH determined by the glass electrode method. This is mainly due to the fact that the pH of the oxygenated haemolysed blood is increased by reduction.

2. The pH of oxygenated whole blood cannot be measured by the hydrogen gas electrode method at the body temperature because at the body temperature the acid change due to glycolysis progresses rapidly. The pH of oxygenated rabbit blood at body temperature can be determined by applying a hydrogen gas electrode to the plasma separated at a room temperature (20°–25°C). Such a measurement on the plasma sample is, however, not applicable to the blood of human and other animals, as the pH of the plasma separated at room temperature and measured at the body temperature is higher than that of the whole blood measured at the same temperature. Accordingly, the glass electrode method is the only one which gives the accurate pH of oxygenated blood at the body temperature.

3. When the plasma of whole blood is separated at a room temperature (10°–15°C) and its pH is compared with that of the same whole blood, both being measured at the body temperature

by the glass electrode method, the former is always higher than the latter. This is true of all the animals examined. The average value of the differences are as follows: 0.08 pH in human blood, 0.10 pH in cat blood, 0.10 pH in dog blood, 0.06 pH in goat blood, 0.06 pH in horse blood, 0.02 pH in rabbit blood, 0.11 pH in cock blood and 0.10 pH in toad blood.

This difference is due to the fact that the temperature coefficient of the pH of whole blood which is negative at a constant total CO_2 content is less, i. e. larger in absolute value, than that of its separated plasma, as haemoglobin displays its powerful buffer action in the former.

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STUDIES ON PROTAMINE (II).

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Mugiline β, the protamine of the sperm of Mugil japonicus, Temminck et Schlegel, as reported in our first paper, contains much arginine, and a relatively large amount of monoamino acids which are soluble in methanol and ethanol. A large amount of a new sample of it was prepared and hydrolysed with sulphuric acid. From the hydrolysate was isolated alanine, valine, and proline, together with arginine. No other basic amino acid was recovered. The molecular proportion of arginine to total monoamino acids was approximately 2:1. Thus mugiline β belongs to the salmine group of the monoprotamines, according to A. Kossel's classification.

Moreover a small quantity of a diketopiperazine, in the form of glittering needles, was discovered in the hydrolysates of mugiline from two different sources. It showed a faintly positive Sasaki's reaction but the ninhydrin test was negative. It was identified as valine anhydride by hydrolysing experiments, by comparing it with the synthetic product, and by elementary analysis. Its $[\alpha]_D^{20}$ is -62.33 and it decomposes at 299° (uncorr.).

In 1907 E. Fischer and G. Schenkel prepared valine anhydride by melting dl-valine. It might therefore be suspected that the valine anhydride in the hydrolysate was formed by prolonged heating of valine in H_2SO_4. Experiments with l-valine by boiling with sulphuric acid, however, did not demonstrate the formation of any anhydride. It seems obvious then, that the valine anhydride was either contained as such or formed from valylvaline, a dipeptide from monoamino acids, by the action of 25 % H_2SO_4 at its boiling temperature. The existence of the latter in the mugiline β molecule is the more provable.

From the results of amino-N determination with the formol-titration method, M. Nelson-Gerhardt (1919) concluded that salmine had a peptide of monoamino acids. E. Gross (1922), and K. Felix with his co-workers (1933) came to the same conclusion with clupeine. However up to the present, no one has isolated such a substance from any protamine.

On the other hand K. Felix and his co-workers (1933) have isolated tetrarginine peptide from clupeine hydrolysate. From these data it is likely that the protamine has, an **-aaaamm-** or a **-mmaaaa-** chain in its molecule together with an **-aamaam-** or an **-amaama-** chain. In these chain formulae, arginine and monoamino acid are denoted by **a** and **m** respectively.

EXPERIMENTAL.

Experiment I.

3.7653 g. of mugiline β sulphate or 2.9509 g. of free protamine was boiled for twenty hours in an oil bath along with 40 ml. of 25 % H_2SO_4. After cooling it was diluted with water up to 200 ml., 2 ml. of it contains 8.8390 mg. N, equivalent to 29.95 % of the free protamine. To the 196 ml. remaining was added an excess of phosphotungstic acid. From the washed precipitates was regenerated a solution of basic amino acid. When this was diluted to 200 ml., 2 ml. of it were found to contain 1.7612 mg. amino- and 7.0993 mg. of total N. Thus, 81.96 % of the total N of mugiline β was precipitated by phosphotungstic acid. Further the proportion of amino-N to total N in the phosphotungstate was 24.81 %. From 150 ml. of the solution of basic amino acid was obtained 4.4997 g. of arginine flavianate or 1.6046 g. of free arginine. Theoretically, from the total N there should have been 1.6545 g. It becomes evident that mugiline β contains 73.98 % arginine and no other basic amino acid.

Experiment II.

Mugiline β sulphate, which corresponds to 131 g. of protamine free from moisture and H_2SO_4, was hydrolysed for 16 hours with 980 ml. of 25 % sulphuric acid in an oil bath. The hydrolysate showed a negative biuret reaction. It was diluted with water until it contained 5% H_2SO_4, after which phosphotungstic acid was added to divide it into two fractions, basic and monoamino acids. Though the basic fraction was investigated carefully by the method of A. Kossel and F. Kutscher to detect the presence of any other basic amino acid than arginine, none could be found. According to Experiment I, this fraction should contain 96.9 g. arginine.

The monoamino acid fraction.

From the filtrate of the phosphotungstate, phosphotungstic acid and H_2SO_4 were removed, dried in vacuo, (weight 30.3 g.) boiled with absolute ethanol, and separated into two fractions; soluble and insoluble in ethanol and examined closely after M. A. B. Brazier's (1930) method.

A. The fraction soluble in ethanol.

The greater part of this was soluble in water but a small portion remained undissolved, these are referred to as **Aa** and **Ab** respectively.

Aa. **Aa** was converted into its Cu salt and boiled with ethanol. The fraction soluble in ethanol, weighed 15.1 g. and corresponded to 11.8 g. of free proline.

Calculated for $C_{10}H_{16}O_4N_2Cu$	Cu 21.79	N 9.60
found	,, 21.25	,, 9.64

From the residue second crop of the copper salt of proline was obtained by boiling with methanol. This crop weighed 0.8 g. or 0.6 g. of free proline.

Calculated for $C_{10}H_{16}O_4N_2Cu$	Cu 21.79	N 9.60
found	,, 20.37	,, 9.74

The Cu salt insoluble in methanol was treated with H_2S and the Cu removed. It weighed 2.3 g. and decomposed at 289°.

Calculated for $C_3H_7NO_2$	C 40.03	H 7.86	N 15.75
found	,, 40.57	,, 7.39	,, 15.99

Ab. The small portion which was soluble in hot ethanol but not soluble in cold water, weighed 0.3 g., was recrystallized from dilute ethanol. It consisted of microscopic white glittering needles which shrunk at 297°, and decomposed at 299° (uncorr.). It showed Sasaki's reaction faintly but no ninhydrin test, and had no amino-N.

3.056 mg. of the substance gave	2.702 mg. H_2O and 6.807 mg. CO_2.		
3.170 ,, ,, ,, ,, ,,	0.399 ml. N_2 at 25° and 763 mm.		
Calculated for $C_{10}H_{18}N_2O_2$	C 60.56	H 9.15	N 14.14
found	,, 60.74	,, 9.89	,, 14.47

The sample when dissolved in glacial acetic acid showed the following rotation:

$$[\alpha]_D^{20°} = \frac{-0.73 \times 100}{0.1464 \times 2 \times 4} = -62.33$$

0.0490 g. of this substance was hydrolysed with 10 ml. of conc. HCl for 25 hours and the HCl was removed in the usual way. It was converted into a Cu salt by boiling with $CuCO_3$, its methanolic extract, when dried, weighed 0.0786 g. instead of the theoretical weight of 0.0731 g.

5.411 mg. of the substance gave	0.437 ml. N_2 at 18° and 765 mm.	
4.462 ,, ,, ,, ,, ,,	0.356 ,, ,, ,, 17° ,, 766 ,,	
4.758 ,, ,, ,, ,, ,,	2.545 mg. $CuSO_4$	
4.963 ,, ,, ,, ,, ,,	2.634 ,, ,,	
Culculated for $C_{10}H_{20}N_2O_4Cu$	Cu 21.49	N 9.47
found	,, 21.30, 21.14	,, 9.53, 9.47

The ethylester of valine was prepared from l-valine in the usual way and free ester was left standing with a little dilute ethanol. Valine anhydride was formed slowly in the form of a small quantity of microscopic needles, glittering like silk. It decomposed at 298°, it was mixed with the foregoing sample which was obtained from the mugiline β hydrolysate, but the decomposition point of this mixture showed no depression.

B. The fraction insoluble in ethanol.

It was converted into its Cu salt, repeatedly boiled with methanol, and divided into two fractions: The soluble **Ba** and the insoluble **Bb**.

Ba. Ba weight 8.8 g. equivalent to 8.0 of free valine. It shrunk at 280° and decomposed at 285°.

Calculated for $C_{10}H_{20}N_2O_4Cu$	Cu 21.49	N 9.47
found	„ 21.69	„ 9.37, 9.52

From the Cu salt, Cu was removed with H_2S and free amino acid was obtained.

Calculated for $C_5H_{11}NO_2$	C 51.28	H 9.48	N 11.96
found	„ 51.41	„ 9.65	„ 11.96

Bb. The insoluble Cu salt in methanol was freed from Cu by means of H_2S, and dried in vacuo. It weighed 3.5 g.

3.917 mg. of the substance gave 5.778 mg. CO_2 and 2.857 mg. H_2O.
4.093 „ „ „ „ „ 0.548 ml. N_2 at 27° and 766 mm.

Calculated for $C_3H_7NO_2$	C 40.03	H 7.86	N 15.75
„ „ $C_5H_{11}NO_2$	„ 51.28	„ 9.48	„ 11.96
found	„ 40.23	„ 8.16	„ 15.34

This substance is probably composed mostly of alanine and the rest, of valine; approximately 3.3 g. alanine and 0.2 g. valine.

The total yields of monoamino acids are as follows.

Proline	12.4 g.	9.5 %	0.11 mol.
Alanine	5.6 „	4.3 „	0.06 „
Valine	8.5 „	6.5 „	0.07 „

This molecular sum 0.24 is approximately half of arginine mol 0.56 or 96.9 g. calculated from the results of Experiment I.

Experiment III.

Another sample of mugiline β sulphate, corresponding to 45 g. moisture and H_2SO_4 free protamine, was hydrolysed with 25 % H_2SO_4 for 20 hours in an oil bath. The hydrolysate was investigated in the same way as in Experiment II and 0.19 g. of valine anhydride was recovered. It showed no ninhydrin test but a faintly positive Sasaki's reaction. It decomposed at 298°.

Calculated for $C_{10}H_{18}N_2O_2$	N 14.14
found	„ 14.23

Experiment IV.

About 0.3 g. of l-valine, dissolved in 30 ml. of 25 % sulphuric acid, was boiled for 20 hours in an oil bath. Two specimens from the solution were taken before and after boiling, and the total N and amino-N were determined. The results were as follows.

	Nitrogen :		Ratio Amino-N : Total-N
	amino	total	
Before boiling	0.9672 mg.	1.1048 mg.	87.55 %
after boiling	0.9828 „	1.0918 „	90.02 „

SUMMARY.

Mugiline β was hydrolysed with H_2SO_4 and 9.5 % proline, 4.3 % alanine, and 6.5 % of valine were obtained. It contained no other basic amino acid than arginine, which amounted to 73.98 %. This was calculated from arginine flavianate which was estimated in a small portion of the sample.

The molecular proportion of arginine and other monoamino acids is approximately 2:1, so that it can be expressed $\mathbf{a}_2\mathbf{m}$ as asserted by A. Kossel, denoting arginine and monoamino acid as **a** and **m** respectively.

In addition, a small amount of valine anhydride was isolated from the hydrolysate, showing that a part of valine probably forms a dipeptide of valine: valylvaline in the molecule of mugiline β.

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STUDIES ON THE CHANGES IN THE PHYSIOLOGICAL CONDITION OF THE NEW-BORN INFANT. III.*

On the renal function, with special reference to the excretion of indigo-carmine and phenolsulphonephthalein.

By

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In earlier reports, Watanabe and Shoji, (1935) and Shoji, (1936) investigated changes in the distribution of urinary nitrogen, the excretion of urinary mineral salts, and acid-base equilibrium in the new-born infant. Their results showed that in the earliest period of life metabolism was largely dependent on the breakdown of the body proteins, relative hunger and incomplete functional activities of the liver and kidneys.

Hitherto, the functions of these two organs, the liver and kidney, in the new-born have been examined in relation to jaundice and presumed to be either incomplete or deficient by urine and blood examinations. The methods, employed at that time cannot be considered today as either satisfactory or accurate.

It has generally been recognized that the tests for urobilin and bilirubin are negative in the urine of the new-born, despite the well-marked jaundice, and some of the investigators, (Gilbert, 1900-1913 etc.) have presumed that the absence of bilirubin in the urine is due to renal dysfunction. Ylppoe (1913) on the other hand, conducted a bilirubin loading test on young and mature dogs and found that the former could excrete relatively large amounts of this substance in the urine. He therefore, presumed that the renal function in the new-born was not incomplete. Nagatake (1932) also examined the fate of *phenolsulphonephthalein* in the blood stream of young and mature rabbits and found that it was removed more quickly in the younger animals. In this work no quantitative estimations of it in the urine were made, and the animals used were also not younger than thirty days from birth. There are, in addition to these, other reports on renal dysfunction in the new-born, as being due to the relatively poor excretion of urine, to the frequent presence of proteins and epithelial plugs, or of uric acid-infarcts in the kidney. However, in subjects under thirty days there has been no positive attempts to determine the real state of affairs actually existing by resorting to loading tests with foreign materials as it has been done in fully mature subjects. Accordingly an attempt was made to observe the changes in the excretory function of the kidneys shortly after birth by testing their power to excrete foreign bodies when introduced into the body.

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* First and second Report: Jika Zasshi, No. **433**, 868, **434**, 990, (1936).

As experimental material young dogs were used and the results showed that shortly after birth the power to excrete *indigo-carmine* and *phenolsulphonephthalein* was poor. As days elapsed however, there was definite improvement and at about the fourth or fifth weeks the excretory condition resembled that seen in adults.

(The experiments in hepatic function will be reported in the succeeding paper, Report IV).

Experimental Materials and Methods of Study.

The young dogs used were all carefully checked as to the actual number of days of age, all being puppies in the suckling stage. Their general characteristics have already been described in Report II so that they will not be repeated.

In collecting urine from these young animals the use of a catheter is impossible owing to the small diameter of the urethra. Hence, when frequent specimens are required it is necessary to make an artificial fistula, but this is better avoided if possible. Fortunately, in these young animals the bladder is situated between the umbilicus and the pubic bones, immediately below the abdominal muscles, so that when required, the urine can be aspirated with a syringe by puncturing in either of the mammary lines. The use of an anaesthetic is unnecessary and the method is to be recommended where one or two collections are needed. The details of the actual procedure used are given under the separate sections of the work.

Experiment 1. Tests with indigo-carmine.

Indigo-carmine is excreted chiefly by the kidneys. It does not form a leuco-derivative, so that in surgery it has been widely used in the examination of diseased kidney function. However, it is not infallible and because of its variability, is not being extensively used today in the tests for the total renal function. Accordingly the tests with this dye have been limited to only a few cases.

A four per cent solution of the dye in normal saline was prepared and 1 ml. of this was injected intravenously into three puppies, each aged thirty days. Within three minutes the urine was faintly coloured. The colour increased at the end of four minutes and by the end of the fifth minute the colour was a deep blue. The skin pigmentation disappeared within two or three hours.

When animals were examined on the day after birth, in all three of them, when a dose of 0.5 ml. was given, the dye appeared in the urine within four or five minutes but the deepening of the colour was not so rapid and the dye could still be seen in the skin and oral mucous membrane the following day. The results definitely pointed to the poorer excretory power of the younger animals.

Experiment 2. Tests with phenolsulphonephthalein.

Phenolsulphonephthalein is excreted chiefly by the kidney tubules. It is a good indicator of the total renal function and as such, has been widely used in functional tests on the kidneys. It has been reported that when a 0.6 per cent solution of the dye is injected into the gluteal muscles of a healthy human adult, the dye appears in the urine within five to ten minutes, and that in the first hour 35 to 60 %, and in the second hour a further 15 to 25 % is passed into the urine. Thus, within two hours after introduction of the dye, 50 % to 85 % of the total is recovered and by the fifth hour or by the sixth hour at the latest, all the dye will have been removed from the body. With a diseased kidney the excretion is not so rapid and the percentages are lower.

In the present work a dose of 2 ml. of a 0.012 % solution of the dye per kg. body weight was injected into the jugular vein. As a standard for comparative colorimetry, 1 to 2 ml. of a 0.006 % solution of the dye made alkaline with 25 % sodium hydroxide solution, was diluted with the urine from healthy puppies of the same age as those in the experiment. This was rendered necessary because the urine in these young animals was decidedly yellow, like that in the human infant. This colour is probably due to excess of urochrome.

In some of the animals the urine was collected from the bladder two to five hours after the introduction of the dye, by aspirating with a syringe according to the method already described. The samples were analysed for their *phenolsulphonephthalein* content by first making them alkaline with 25 % NaOH solution and comparing the colour with the standard.

In others, an urinary fistula was made under anaesthesia, and five to six hours later the dye was introduced and the time of the first appearance of the dye was noted. The amount excreted at the fixed times was also noted and estimated. By this method the excretion tended on the whole to be poorer but the differences were too slight for it to be considered separately from the previous method.

An attempt was next made to increase the total volume of urine excreted by introducing about 20 ml. of physiological saline solution subcutaneously or into the stomach by means of a Nelaton's catheter, and to collect the urine at repeated short intervals. The attempt failed however, especially in younger puppies, due to the extremely poor excretion of urine and dye. In these cases attention was paid to the first appearance of the dye and to the percentage amounts excreted within the second and fifth hours. After the fifth hour the dye was still excreted in all cases but the amounts became less with the age of the animals.

RESULTS.

1. Animals in the first week of life. (See Table I).

The earliest time of appearance of the dye was twelve minutes and the latest fourty minutes. In both cases the appearance was markedly delayed.

TABLE I.

Phenolsulphonephthalein (1)

Days after birth	Body weight in grams.	Amount injected in mgms.	Appeared after minutes.	Amount excreted in percentages.		
				2nd hour	5th hour	after 5 hours.*
1	125	0.030		17.91		
1	175	0.042		19.56		
1	292	0.072			23.26	
1	310	0.072	20	15.50	23.84	+
1	322	0.078	30	11.27	16.44	+
1	335	0.084		21.76		
1	335	0.084			32.12	
1	340	0.084			17.26	
1	350	0.084	40	12.96	20.53	+
1	360	0.084		19.04		
2	345	0.084		13.54		
2	360	0.084		17.73		
2	365	0.084		17.19		
2	365	0.084			37.32	
2	370	0.084			43.01	
2	385	0.084			47.12	
3	226	0.060		28.55		
6	377	0.090	24	12.37	36.43	+
6	410	0.096	19	23.94	47.73	+
6	412	0.096	37	14.31	18.62	+
6	527	0.132	12	30.73	52.68	+
Average				18.43	32.03	

* (+) positive, (±) weakly positive.

The percentages excreted by the end of the second hour were a minimum of 11.27 % and a maximum of 30.73 % with an average of 18.43 %. These too, were noticeably poor. The figures at the end of the fifth hour were equally low and less than that excreted by older animals.

2. *Animals in the second to third weeks of life (See Table II).*

Even with these animals, though in two cases aged 21 days, the dye could be demonstrated in the seventh and nineth minutes, there was one which required twenty minutes, so that the excretion could not be considered good. The total amount, of the dye excreted in two hours was minimum: 22.66 %, maximum: 51.63 %, with an average of 35.81 %. In five hours from 46.35 % to 71.14 %, averaging 57.34 % were excreted.

TABLE II.

Phenolsulphonephthalein (2)

Days after birth	Body weight grams.	Amount excreted mgms.	Appeared after minutes	Amount excreted in percent.			
				1st hour	2nd hour	5th hour	after 5 hours.
14	357	0.084			36.82	60.09	
14	397	0.096			28.76	49.59	
14	555	0.132	14		28.97		
14	640	0.156	22		30.38		
14	970	0.228			34.80	52.57	
14	1080	0.240			40.78	59.41	
15	700	0.168			22.66	46.37	+
15	700	0.168			26.03	49.14	+
15	780	0.192			48.39	79.67	+
21	1000	0.240	7	18.54	32.79	53.80	+
21	1050	0.252	20	15.53	31.24	46.35	+
21	1140	0.264	9	25.16	41.20	79.22	+
21	1300	0.312			71.63	71.14	
21	1400	0.336			46.89	69.68	
Average				19.74	35.81	57.34	

3. Animals in the fourth to fifth weeks of life. (See Table III).

With these animals the dye could be demonstrated in the majority of cases as early as the sixth and seventh minutes and showed a decided improvement on the previous two sets of younger animals. The lowest percentage excreted by the end of the second hour was 38.99 % and the highest 68.14 % with an average of 53.00 %, so that the figures approximated those for healthy human adults. The amounts excreted by the end of the fifth hour were also high as shown in the table.

TABLE III.

Phenolsulphonephthalein (3)

Days after birth	Body weight grams	Amount injected mgms.	Appeared after minutes	Amount excreted in percentages.				
				30 min.	1st hour	2nd hour	5th hour	after 5 hours.
28	656	0.156				51.40	62.93	
28	835	0.204				58.11	65.06	
30	565	0.132	14		21.11	38.99	62.32	+
30	615	0.144	6		26.77	43.16	64.30	+
30	1780	0.432	7		42.71	68.14	81.71	±
34	1000	0.240				51.10	59.29	
34	1100	0.264				53.51	65.75	
34	1130	0.264				60.55	59.03	
35	860	0.204	6					
35	950	0.228	7			46.30		
35	1020	0.240				50.14	56.86	
35	1050	0.240				48.46	56.16	
36	1700	0.420	7	38.02	50.64	62.44		
36	1850	0.420	7	32.61	47.11	56.68		
Average				35.32	37.67	53.00	63.34	

SUMMARY.

From the foregoing it will be seen that the excretory function in the animals at the fourth to the fifth weeks of life approximated that in the full-grown adult, both as regards the first appearance of the dyes in the urine and the percentage excreted in the second and the fifth hours. Animals slightly younger, that is, those in the second and third weeks showed that they were still below the normal in their excretory function. Animals younger than these, in the first week after birth, were still more handicapped. (See Chart I and II). In regard to the times necessary for excreting 50 % of the total dye introduced, those animals in the fourth to fifth weeks required only 2 hours; those in the second to third weeks, five hours; while those in the first week required more than five hours.

Chart I. Percentages of phenolsulphonephthalein excreted in first 2 hours.

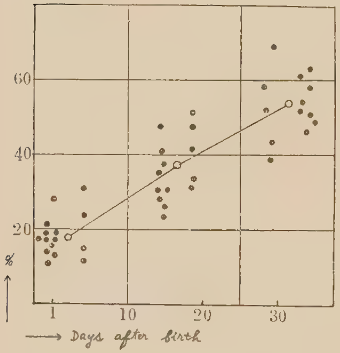
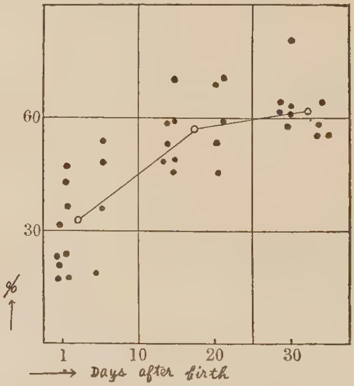


Chart II. Percentages of phenolsulphonephthalein excreted in 5 hours.



CONCLUSION.

From the results of these tests on the renal function with the two dyes, *indigo-carmine and phenolsulphonephthalein*, it is evident that the kidneys of puppies, shortly after birth, had not attained their fully excretory function. However, this function improved with the age of the animals until the period of about the fourth or fifth week of life at which time the condition was approximately that of the mature adult.

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STUDIES ON THE CHANGES IN THE PHYSIOLOGICAL CONDITION OF THE NEW-BORN INFANT. IV.

On the Liver Function, with special reference to the Excretions of Azorubine S, Congo Red and Santonin dyes.*

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I. Introduction.

To date, there has not been any single satisfactory method of investigation of the total functions of the liver. This is probably due not merely to the diverse functional activities of this organ but to its intimate relationship to the activities of other systems. Accordingly, varied methods of investigating these functions have to be devised when different objects are in view.

On going through the literature on the functional tests of the liver in the new-born infant, only a few experiments on sugar metabolism by means of loading tests ate found, viz. that of Heynemann (1914), Duzar and Hensch (1914), Styrikowitsch (1927), Naito (1931), Winter (1933). These were conducted however, over short periods of time and no attempt was made to study the changes in the functional activity that accompany the growth of the animals. Comparison of the abilities of the reticulo-endothelial system in absorbing *congo red* in the young and fully mature rabbits has been made by Ide (1930), Hamada (1930), Nagatake (1932), Miyoshi (1936). The condition has however, not been investigated in animals from birth to their thirtieth day of life. Linzenmeier (1922) reported that

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* The gist of this report was communicated at the 31st Annual Meeting of the Formosa Medical Association, November, 1936.

Widal's "Crise hemoclasique" test is positive in new-born infants and Akiyama (1924) showed the presence of alimentary leucopenia. However, opinion is divided as to whether these conditions are due to liver dysfunction or vagotonia. The hepatic function in the new-born has been the subject of further investigation in connection with jaundice and considered as incomplete or immature by one or two examinations of meconium, urine and blood; viz. Bassi and Chauffard (1888), Minkowski (1886), Simon (1911), Nakamura (1936).

Kondo (1926) examined the changes in the amount of blood bilirubin and lipase after birth in dogs and advocated that the jaundice of new-born infants was unrelated to incomplete hepatic function.

In the experiments to be reported in this paper and in succeeding ones, attempts have been made to investigate the functions of the liver in young dogs by means of loading tests. The methods which have been chosen, seemed to have afforded the only means of investigating fully the various functional condition of the liver.

The tests with *azorubine S*, *congo red* and *sodium santonate* showed that the function of the liver in absorbing and excreting foreign substances, and in the case of sodium santonate of bringing about a chemical change, was noticeably poor in dogs of 1—2 to 5—6 days old. An improvement was seen in older dogs of from 10—20 days old, while in those one month old, the function practically attained the state found in mature adults.

II. EXPERIMENTAL.

Experiment 1. On the excretion of azorubine S in the urine.

A. According to Tada and Nakashima (1923), in healthy adults this dye is excreted mainly into the bile and only about 4 % finds its way into the urine. However, with a dysfunction of the liver, the opposite is observed, and the kidneys excrete a large portion of the dye so that this test has been recommended as of definite value in clinical practice. Since then, Kimura (1926), Akiyama (1926), Gen and Kin (1930), Chinen

(1931), Nakagawa and Mitani (1933), have found that an increase in the renal excretion of this dye was indicative of the extent of the pathological changes in the liver. Fenstermann (1926) believes this method to be superior to any that has hitherto been used.

In the present work an attempt has been made to estimate indirectly the hepatic function in young dogs by the introduction of *azorubine S* and noting the amounts excreted in the urine. Consideration was paid to the fact that if the renal function was poorer than that of the liver, the results would fall short of that expected.

B. Preliminary experiment. A dose of 1 % solution of the dye in normal saline per kg. bodyweight was injected into the jugular vein of 2 young dogs, aged 33 days, and the time required for the complete excretion of the dye in the urine noted. The results as indicated in Table I, showed that within 1 to 2 hours, the major portion of the dye that should be excreted with the urine passed through the kidneys, so that the amount excreted after that time was extremely small.

TABLE I.

Azorubine S (1)

Exp.	Body weight in grams	Amount injected in mgms.	Amount excreted (at separate hours)				Amount excreted in the first 2 hours (%)
			1	2	3	4	
No. 1	1160	23	2.45	0.35	0.14	0.00	2.80
No. 2	850	17	1.14	0.89	0.41	0.25	2.03

Accordingly, the method of estimating the quantities of dye excreted in the urine in the first two hours, or in some cases the first four hours, was resorted to. By this method it was not only possible to have an idea of the total dye excreted but the necessity of frequent collections with accompanying discomfort to the animals was avoided. The collected urine was then estimated for its dye content by the method of Tada (1923) against a standard solution. The standard was prepared from the urine of normal animals of the same age as those used in the experiment.

C. Results. The results as indicated in Chart 1 and Table II showed that the animals aged 1 to 3 days after birth passed more than 3.5 % of the dye in the first two hours. With older animals, from 5 to 6 days, in a few cases there were indications of a drop in the percentage excreted. In animals aged 9 to 10 days, in more than half of the cases the percentage excreted was less than 3.5 %. With still older animals, aged 18—25 days, or 30—43 days, nearly all of them showed a lower output than 3.5 %. The amounts excreted in the second to the fourth hours showed similar tendencies in the few cases examined.

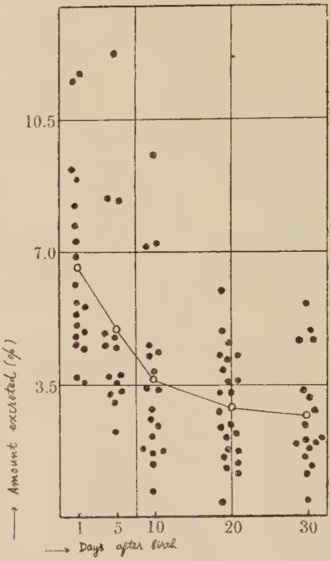


Chart 1 Azorubine S.

TABLE II.

Azorubine S. (2)

Days after birth	Body weight in grams.	Amount injected in mgms.	Amount excreted in percentages.	
			0-2nd hour.	2-4th hour.
1	275	5.5	3.65	
1	325	6.5	6.84	0.21
1	355	7.0	4.58	0.19
1	359	7.0	—	0.15
1	382	7.5	4 82	0.43
1	385	7.5	6.17	1.23
2	260	5.0	11.55	0.13
2	327	6.5	11.72	1.42
2	380	7.5	8.25	1.23
2	392	8.0	5.13	0.36

D. Discussion.

The method employed contrary to the test with *sodium santonate* depends upon the healthy functional activity of the

Days after birth	Body weight in grams.	Amount injected in mgms.	Amount excreted in percentages.	
			0-2nd hour,	2-4th hour.
3	270	5.5	5.54	
3	277	5.5	5.73	
3	325	6.5	5.43	
3	330	6.5	3.59	
3	342	7.0	4.88	
3	350	7.0	9.20	
3	385	7.5	8.97	
3	397	8.0	7.32	
3	410	8.0	7.73	
3	420	8.5	4.47	
Average			6.61	0.59
5	367	7.0	3.56	
5	370	7.0	3.06	
5	414	8.0	4.53	
5	470	9.5	3.33	
6	295	6.0	4.56	
6	349	7.0	12.30	
6	377	7.5	8.37	
6	407	8.0	3.74	
6	420	8.5	2.24	
6	442	9.0	4.89	
6	472	9.5	4.75	
6	475	9.5	8.37	
6	482	9.5	3.77	
6	515	10.0	3.33	
Average			5.06	
9	295	6.0	1.68	
9	320	6.5	0.69	
9	360	7.0	3.38	
9	400	8.0	1.73	
9	405	8.0	2.82	
9	425	8.5	1.44	
9	432	8.5	1.74	
9	492	10.0	3.81	
9	520	10.0	3.56	
10	367	7.0	7.62	
10	405	8.0	2.10	
10	467	9.0	4.20	
10	470	9.5	4.50	
10	480	9.5	2.58	
10	522	10.0	9.50	
10	572	11.5	3.41	0.58
10	585	11.5	4.35	
10	620	12.5	7.26	
10	760	15.0	2.42	0.67
Average			3.62	

Days after birth	Body weight in grams.	Amount injected in magms.	Amount excreted in percentages	
			0-2nd hour.	2-4th hour.
18	485	9.5	3.96	
18	560	11.0	4.95	
18	740	15.0	3.33	
18	750	15.0	5.93	
20	470	9.5	0.36	
20	550	11.0	4.61	
20	560	11.0	2.09	0.16
20	590	12.0	4.22	0.07
20	600	12.0	3.26	
20	605	12.0	1.34	0.24
20	610	12.0	1.73	0.51
20	619	12.0	2.24	0.46
20	630	13.0	2.78	0.13
20	650	13.0	3.54	
20	650	13.0	4.26	
20	650	13.0	2.36	0.25
20	880	18.0	4.28	
22	950	20.0	1.43	
22	1000	20.0	1.74	
23	1000	20.0	1.25	
23	1000	20.0	1.61	
25	627	13.0	3.56	
25	650	13.0	1.23	
25	790	16.0	2.36	
Average			2.85	0.26
30	610	12.0	2.00	
30	615	12.0	1.61	
30	650	13.0	1.97	
30	700	14.0	1.19	
30	700	14.0	2.49	0.10
30	900	18.0	1.97	0.04
30	950	19.0	3.35	0.06
30	960	20.0	4.74	0.09
30	1000	20.0	5.63	0.06
30	1020	20.0	4.74	0.09
30	1030	20.0	4.59	0.09
30	1100	22.0	0.45	
30	1330	26.5	1.32	
30	1650	33.0	1.91	
33	850	17.0	2.03	
33	1160	23.0	2.79	
43	1180	23.5	3.20	
Average			2.70	0.07

kidneys. In Report III it was shown that the renal function in young dogs, was in a poorly developed state in the very young animals, but in spite of this, the present results showed that the percentages of *azorubine S* excreted in the urine by animals of five to six days old, were in the majority of cases more than 3.5 %, while older animals of ten days or more showed figures of less than 3.5 %. From this it seems clear that in these young animals there exists a corresponding dysfunction of the liver. If the kidneys were functioning normally, it is probable that greater differences in the percentages renally excreted might be obtained.

Experiment II. On the retention of congo red in serum.

A. When *congo red* is injected into the blood it is taken up quickly by the lining endothelial cells. Most of the dye is then passed into the bile and only a small portion of it finds its way into the urine. Adler and Reimann (1925) showed that since the dye is taken up by the reticulo-endothelial system, the dye was a good test material for investigating the general functional activities of this system. Since then the dye has been extensively used in both clinical and laboratory investigations. Other investigators, Ide (1930), Miyoshi (1936), et al. recognized the *congo red* method of Adler and Reimann, but others Schellong (1928), Ninomiya (1928) and Takede (1930), denied the value of this method, as the excretion of this dye depends also to a large extent on the relative functional activity of the liver cells.

In the present work the dye was used solely for testing the functional activity of the liver, as the kidneys were not connected with its excretion. It was hoped that an idea of the functions of the reticulo-endothothelial system in the period immediately after birth could also be gained from these experiments.

B. Only young dogs of proven age record were used. Before the experiment these puppies were isolated from their mothers for 6 to 6½ hours. Two ml. of a 1 % solution in normal saline per kg. of bodyweight were used. The dye solution was injected into the jugular vein and at the fourth and sixtieth minutes after introduction of the dye blood, was temoved from the opposite jugular vein. The serum was then collected by centrifugalization and diluted 5–6 to 10 times with normal saline. The amount of *congo red* in a definite volume was then estimated by comparing the color with a standard.

C. Experimental Results.

(a). Change in ratio. Adler and Reimann worked out a system of indices by setting the amount of congo red contained in the serum collected at the 4th minute as standard. This can be done satisfactorily in man (Griesbach (1921) and others) but its use in the small laboratory animals especially in rabbits, has been criticised by Muta (1931), and Shinagawa (1932).

Accordingly no serious consideration was paid to the indices worked out. The indices were obtained however, by the methods hitherto used and the results are tabulated in table III.

With dogs aged 1 to 2 days the *congo red* index was in the majority of cases 40–50 %. With older animals (30 days after birth) the index was less than 30 % so that the differences

TABLE III.

Congo red.

Days after birth	Body weight in grams.	Amount injected in mgms.	Amount remaining in mg %		Congo red Index
			4th min.	60th min.	
1	302	6	9.265	4.719	50.93
1	340	7	8.828	3.741	42.38
1	350	7	7.384	3.031	41.05
1	372	7	6.693	3.322	49.63
1	395	8	6.316	3.228	51.11
1	410	8	7.251	2.225	30.69
2	337	7	5.502	2.831	51.45
2	362	7	5.457	2.957	54.19
2	370	7	9.972	4.082	40.93
2	370	7	8.716	4.291	49.23
2	390	8	8.240	4.204	51.02
2	392	7	5.531	2.703	48.87
Average			7.429	3.444	46.79
5	185	5	7.812	2.888	36.97
5	225	5	6.101	2.684	43.99
5	410	8	5.605	2.228	39.75
5	460	9	5.030	2.389	47.50
6	460	9	6.944	1.825	26.28
6	475	9	5.922	1.398	23.61
6	495	10	5.650	2.347	41.54
Average			6.152	2.251	37.09

Days after birth	Body weight in grams.	Amount injected in mgms.	Amount remaining in mg %		Congo red Index
			4th min.	60th min.	
10	522	10	4.202	2.002	47.64
10	562	11	3.749	1.593	42.49
10	635	13	3.423	1.142	33.39
10	675	13	3.578	1.459	40.78
11	535	11	4.010	1.079	26.91
11	575	12	6.083	0.947	15.57
11	577	12	4.426	1.074	24.27
11	577	12	4.945	1.151	23.28
11	612	13	3.663	1.408	38.44
11	645	13	3.391	1.069	31.52
11	650	13	1.722	0.458	26.60
Average			3.926	1.216	29.48
19	587	12	2.941	1.126	38.29
19	670	13	3.385	1.105	32.64
20	420	8	2.480	1.004	40.48
20	437	9	3.020	1.077	35.66
20	1200	24	1.614	0.321	19.89
20	1280	26	2.183	0.466	21.35
Average			2.604	0.850	31.38
27	615	12	2.737	0.673	24.59
27	690	14	2.365	0.546	23.08
27	800	16	2.756	0.510	18.51
29	860	17	2.636	0.816	30.96
29	930	19	2.339	0.695	29.71
30	910	18	2.048	0.820	40.04
30	920	18	2.511	0.663	26.40
30	1140	23	1.884	0.487	25.85
30	1550	31	1.405	0.331	23.56
31	1350	27	1.118	0.120	10.73
31	1420	28	1.230	0.192	15.61
Average			2.093	9.532	24.45
60	3600	50	0.800	0.125	15.63
60	4000	50	0.546	0.106	19.41

were well marked. The indices for animals of 5, 10, or 20 days old lie almost between these figures. See Chart 2.

(*b*). *Changes in concentration of the dye in sera at the fourth minute.*

As indicated in Chart 3 the concentration of *congo red* at the fourth minute after injection of the dye was 5.5 mg. % to 10.0 mg. %. The average was 7.43 mg. % for animals of 1

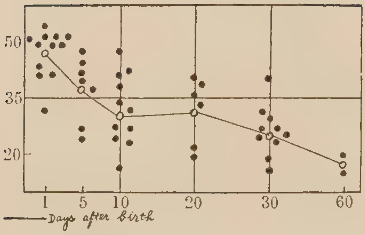


Chart 2.
Congo red index.

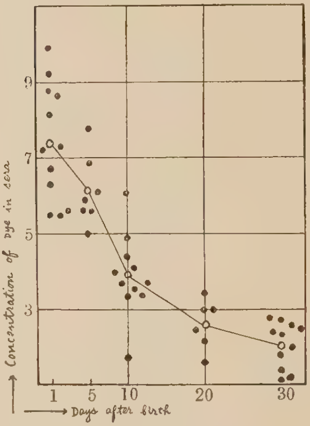


Chart 3.
Concentration of Congo red at the 4th min.

to 2 days old; that for animals 5 to 6 days was 5.0 mg. % to 7.8 mg. %, with an average of 6.15 mg. % so that in both cases very high figures were shown. In those aged 10 days, the figures showed a minimum of 1.72 mg. %, a maximum of 6.1 mg. % with an average of 3.93 mg. %. With animals 20 days old, the figures were 1.6 mg. %, and 3.4 mg. %, with an average of 2.6 mg. %. For those thirty days old the figures were still lower with a minimum of 1.1 mg. %, a maximum of 2.8 mg. % and an average of 2.09 mg. %. Thus it is clear that the figures showed a gradual decline.

(c). *Changes in the concentration of dye in the sera at the sixtieth minute.*

As in the previous results the concentration of the dye in dogs 1 to 2 days old was very high and it gradually decreased with the age of the animals. See Chart 4. However, contrary to the previous results where the figures for animals of from 1—2 days to 5—6 days were noticeably higher than for animals 10 days old, at the sixtieth minute those for animals 5—6 days old already showed a decline when compared with those 1—2 days old.

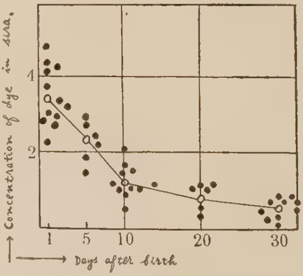


Chart 4. Concentration of Congo red at the 60th min.

From the fact that the absorption of dye by these animals in the 5—6 days of life is still in a poor condition especially in the early stages of introduction of the dye (4th minute) but that the ability improves in the later stages (60th minute), both the absorptive or retentive and excretory function in these animals were delayed. With the groups of older animals the results for the 4th and 60th minutes ran more or less parallel, and showed a tendency for the absorption of dye to improve with the age of the animals.

Experiment III. Tests with santonin dyes.

A. When *santonin* is administered orally or its sodium salt is introduced parentally, its conversion or decomposition product is excreted in the urine and in the alimentary canal. Its presence can readily be detected by the appearance of a red colour with alkali. This substance will be called "*santonin-dye*" for the sake of convenience.

Ishikawa (1926) and Katoh (1928) decided that this substance is the result of a chemical change brought about in the *santonin* by the liver. Hence, by investigating the time of the appearance of the dye in the urine it is possible to estimate the function of the liver. Ono, Yoshida and Yashiro (1928) and Shimomura (1931) confirmed the superiority of this method as a test of liver function.

The advantages of this test lie in (1) that when the liver is in a diseased state or when the liver cells are damaged by the so-called "liver-toxins", or when the reticulo-endothelial system is completely blocked the appearance of this dye in the urine is hindered, (2) when 1 ml. of a 10 % solution of *sodium santonate* is injected intravenously in man the dye appears in the urine as early as the 6—9th minutes, in rabbits by the 18—23rd minutes, and in dogs by the 15—20th minutes (intramuscular), and the excretion of the dye seems to be unaffected by any inflammation of the kidneys, (3) the method of demonstrating the dye is simple and merely requires the addition of a drop or two of 10 % caustic soda or potash into a small volume of urine when the characteristic red color manifests itself. Where the amount of dye is small the addition of a little amyl-alcohol followed by shaking will help to manifest its presence.

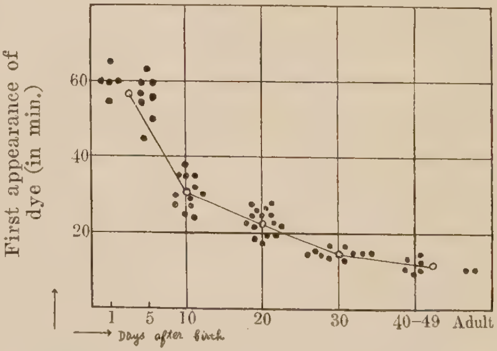
The experiments to follow were accordingly undertaken to find out the speed with which the *santonin dye* is produced in young dogs, and if the speed is poor, when conditions approximate those in the mature adult. The work was of interest not merely from a chromodiagnostic view in the investigation of the excretory function but it promised to present some idea of the biochemical functions of the liver.

B. Method of experiment and results.

The material and method of collection of urine were generally as in Report III. However, here 20 ml. of normal saline was introduced into the stomach by means of a Nelaton's catheter 30—60 minutes before the experiments and in order to hold the urine, the tip of the penis in males and the base of of the vulva in females were tightly secured with threads. *Sodium santonate* was obtained in the form of *Drasmin* prepared by the Shionogi Company and 1 ml. of a 5 % solution was injected into the jugular vein. In the case of animals 1 to 2 days old a 10 % solution was used, for reason to be given later.

As a preliminary procedure it was necessary to determine the approximate times of the first appearance of the dye in the urine in the different sets of animals. This was done by disinfecting the anterior abdominal wall and aspirating small quantities of urine (about 0.5 ml.) at about 3 or 4 minutes' intervals. No anaesthetics were given. The urine was treated with 10 % caustic potash, amyl-alcohol was added and the mixture shaken.

Chart 5. Rate of production of santonin dye.



The results which are indicated in Table IV and Chart 5 showed that for animals 30 days old the minimum time of appearance of the dye in 10 cases was 14 minutes, the maximum time 17 minutes and in half the cases 15 minutes. For animals 40—49 days old, in 6 cases, and for animals a year old, in two cases the times were from 10 to 15 minutes and were similar to the times for mature adults. (These results show slightly more rapid excretion than in Ishikawa's work, where intramuscular injections were employed.)

For animals 9—10 days old, demonstration of dye in the urine could be made as early as the 24th or 25th minutes. There was one however which required 38 minutes and in the majority of cases it was in the neighbourhood of 30 minutes. For animals 19—20 days old the shortest time was 18 minutes and the longest 28 minutes with an average of 23 minutes, and the figures were still higher than for older animals. The intensity of color was more marked in these latter animals.

For animals 1—2 days old, the preliminary experiments showed that excretion was markedly delayed and the concentration of the dyes so low that it was difficult to detect them. Accordingly, to increase the amount injected, 1 ml. of a 10 % solution of *Drasmin* was introduced and for accelerating the excretion 10 ml. of water was administered twice into the stomach before the experiments. Thirty minutes after the injection of the test material 5 or 6 ml. of saline was introduced through the jugular vein. In spite of this, the time of appearance of dye in the urine was prolonged as much as 45—65 minutes in these animals of 1—2 days old and in older ones of 5—6 days old. The concentration of dye was also low and in many cases it was difficult to demonstrate on account of the light color. In the Table the symbol (±) implies that the intensity of the colour was not increased but remained the same as in the preceding sample.

TABLE IV.

Santonin dye.

Days after birth	Body weight in grams.	First appearance (in min.)	Degree of reaction
1	337	65	±
1	250	60	±

Days after birth	Body weight in grams.	First appearance (in min.)	Degree of reaction
1	285	60	+
1	255	55	+
1	289	50	neg.
2	300	60	±
2	350	35	neg.
5	442	63	±
5	415	60	+
6	580	60	+
5	470	57	+
5	405	56	±
5	350	55	±
6	360	50	±
6	450	45	+
6	295	30	neg.
6	347	30	neg.
6	370	25	neg.
Average for positive cases		about 57 minutes.	
10	472	38	+
9	540	35	+
11	700	35	+
10	620	35	+
9	582	32	±
10	410	30	+
10	520	30	+
10	460	30	+
9	550	28	+
9	500	27	+
10	510	25	+
10	520	24	+
9	285	20	neg.
15	420	15	neg.
Average for positive cases		about 30 minutes.	
19	820	28	+
20	640	28	+
19	950	27	+
20	800	26	+
20	775	25	+
19	840	25	+
20	720	25	+
19	850	23	+
20	900	23	+
19	610	22	+
20	750	22	+
19	850	20	+
19	890	20	+
19	820	19	+
19	970	19	±
19	1000	18	±
Average for positive cases		about 23 minutes.	

Days after birth	Body weight in grams.	First appearance (in min.)	Degree of reaction
30	1100	17	+
30	1650	17	+
30	700	16	+
30	615	15	±
30	880	15	±
30	900	15	+
30	1330	15	+
30	1450	15	+
30	610	14	+
30	650	14	+
Average for positive cases		about 15 minutes.	
40	2060	12	+
40	1260	14	+
40	1100	13	+
43	2750	10	+
43	1180	12	+
49	1850	15	+
Adult	12000	11	+
Adult	10000	11	+
Average for positive cases		about 12 minutes.	

Note: (+) positive means first appearance of the dye at stated times.
(neg.) negative. The dye could not be demonstrated at the stated times. Further examination was abandoned owing to insufficiency of urine.

III. Summary and Conclusion.

1. The excretion in percentages of *azorubine S* after its injection into the body was more in dogs 1—3 days old when compared with older animals of 5—6 days. The figures for animals of 9—10 days and 20—30 days were still lower.

2. After intravenous injections of *congo red* the amounts present in the sera at the 4th minute were very high in animals of 1—2 days to 5—6 days old, but showed gradually decreasing figures for animals 10, 20 and 30 days old.

With the 60th minute samples the amounts in these 1—2 days old puppies were very high, but with the older animals the figures decreased proportionately. The ratios also showed corresponding changes.

3. When *sodium santonate* was injected into the jugular vein of dogs, the *santonin dye* appeared in the urine of animals 1—2 days to 5—6 days old at the 50th—60th minutes, in animals 10 days old at the 30th minute, in those 20 days old at about the 23rd minute, and in animals 30—40 days old at the 12th to 15th minutes.

From these results, the functions of the liver and the reticulo-endothelial system in young dogs did not seem to be absolutly, but relatively undeveloped. This was most marked in the younger animals aged 1—2 days to 5—6 days but the condition improved slowly with the lapse of days (10—20) and in animals 30 days old, practically full function was attained.

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SYNTHESE DER γ-METHYLAMINO-β-OXY-BUTTERSÄURE UND γ-METHYLGUANIDO-β-OXY-BUTTERSÄURE.

Von

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Hinsichtlich der Herkunft des Kreatins resp. Kreatinins sind fast alle Möglichkeiten gedacht und von verschiedenen Seiten experimentell untersucht. Unter anderen haben K. Thomas und seine Mitarbeiter (1918-1922) als die mit dem Kreatin im engsten Zusammenhang stehende Substanz Methylarginin und die Methylguanidylsäuren mit gerader Anzahl von C-Atomen, wie γ-Methylguanidobuttersäure und ε-Methylguanidocapronsäure, synthetisiert und ausführliche Fütterungsversuche dieser Verbindungen mit Kaninchen angestellt. Es ist trotzdem nicht gelungen, festzustellen, dass das Kreatin aus diesen Verbindungen entsteht.

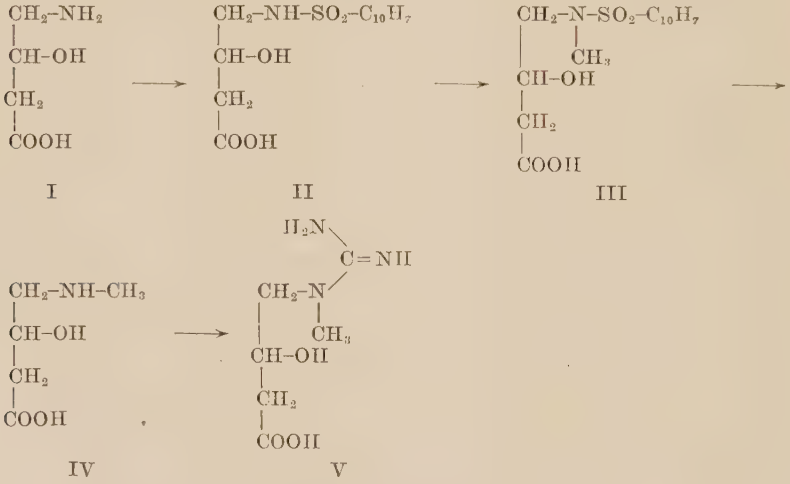
Als eine bisher nicht berücksichtigte denkbare Möglichkeit der Kreatinbildung kann nun die γ-Amino-β-oxy-buttersäure in Frage kommen. Bei dieser Aminosäure ist genau dieselbe Nachbarstellung der Amino- und Hydroxylgruppe vorhanden, wie beim Adrenalin und Cholin. Die N-Methylierung dieser Atomgruppierung kann vielleicht im lebenden Organismus leichter möglich sein.

Es würde somit durch Methylierung aus γ-Amino-β-oxybuttersäure γ-Methylamino-β-oxy-buttersäure entstehen. Dann wird die NH-Gruppe mit einer Guanido-gruppe substituiert—sie kann natürlich auch vorausgehen—und schliesslich folgt Oxydation am β-Kohlenstoffatom.

Zwecks Erklärung dieser Vermutung habe ich als Material der Fütterungsversuche γ-Methylamino-β-oxy-buttersäure und γ-

Methylguanido-β-oxy-buttersäure synthetisch dargestellt.

Beide Verbindungen sind bisher noch unbekannt, ich habe sie über die Naphthalinsulfoverbindung der γ-Amino-β-oxy-buttersäure hergestellt. Durch Einführung einer Sulfosäure in die endständige Aminogruppe, die Methylierung der Sulfamidgruppe und nachfolgende Abspaltung musste ich zu γ-Methylamino-β-oxy-buttersäure (IV) gelangen. Gelang die Kondensation dieser Säure mit Cyanamid, so musste sich die γ-Methylguanido-β-oxy-buttersäure (V) synthetisch auf eindeutigem Wege bereiten lassen. Meine Erwartungen wurden erfüllt. Den Weg der Synthese kennzeichnet die nachstehende Formelreihe.



$$CH_2(NH_2)-CH(OH)-CH_2-COOH \;(\mathrm{I}) \longrightarrow CH_2(NH-SO_2-C_{10}H_7)-CH(OH)-CH_2-COOH \;(\mathrm{II}) \longrightarrow CH_2(N(CH_3)-SO_2-C_{10}H_7)-CH(OH)-CH_2-COOH \;(\mathrm{III}) \longrightarrow$$

$$CH_2(NH-CH_3)-CH(OH)-CH_2-COOH \;(\mathrm{IV}) \longrightarrow CH_2(N(CH_3)-C(=NH)-NH_2)-CH(OH)-CH_2-COOH \;(\mathrm{V})$$

VERSUCHSTEIL.

I. β-Naphthalinsulfonyl-γ-methylamino-β-oxy-buttersäure (III) $C_{10}H_7-SO_2-N(CH_3)-CH_2-CH(OH)-CH_2-COOH$.

Die Methylierung erfolgt quantitativ mit Dimethylsulfat in natronalkalischer Lösung. 6,0 g β-Naphthalinsulfonyl-γ-amino-β-oxy-buttersäure, die nach einem von Fukagawa (1935) ausgearbeiteten Verfahren leicht herzustellen ist, werden in 10 ccm 5 n-Natronlauge gelöst, und mit 3,8 g Dimethylsulfat 3 Stunden auf der Maschine kräftig geschüttelt, dann auf dem Wasserbade 2 ½ Stunden erwärmt. Nach dem Abkühlen wird das Reaktionsprodukt mit überschüssiger Salzsäure ausgefällt, es erstarrt nach einigen Wochen im Eisschrank. Die erstarrte Masse wird in starker Kaliumcarbonatlösung aufgenommen, mit Salzsäure wieder gefällt und im Eisschrank stehen gelassen. Nach einigen Tagen wird abgesaugt und mit Eiswasser ausgewaschen. Ausbeute 6,0 g = 96%. Aus alkoholhaltigem Wasser krystallisiert die Verbindung vollständig in schönen Tafeln oder Blättchen aus, die bei 138–139° schmelzen.

0,0727 g Substanz verbrauchen 2,25 ccm n/10-H_2SO_4.
0,0486 g „ gaben 0,0992 g CO_2 und 0,0223 g H_2O.
0,0506 g „ „ 0,1028 g CO_2 und 0,0232 g H_2O.

		C	H	N
Für $C_{15}H_{17}O_5NS$.	Ber.:	55,70%	5,30%	4,33%.
	Gef.:	55,66	5,45	4,33
		55,40	5,12	

Sie ist leicht löslich in heissem Wasser, sehr leicht löslich in heissem Alkohol, löslich in heissem Benzol und wenig löslich in kaltem Wasser.

II. *γ-Methylamino-β-oxy-buttersäure* (IV)
$NH(CH_3)-CH_2\cdot CH(OH)-CH_2-COOH$.

Die Abspaltung der Naphthalinsulfosäure erfolgte mit Salzsäure. Zu dem Zweck wurden 58 g reiner Naphthalinsulfonyl-γ-methylamino-β-oxy-buttersäure vom Schmelzp. 138–139° mit dem achtfachen Volumen 20%iger Salzsäure im Ölbad 42 Stunden im Sieden gehalten, aus der klaren Lösung wurde die Naphthalinsulfosäure durch 63 stündige Extraktion mit Äther im Suto-Kumagawaschen Extraktionsapparat entfernt, darauf durch wiederholtes Einengen im Vakuum und Wiederaufnehmen mit Wasser ein grosser Teil der überschüssigen Salzsäure, der Rest durch aufeinanderfolgende Behandlung mit Bleicarbonat, Schwefelwasserstoff, Silbercarbonat und wieder Schwefelwasserstoff weggebracht. Die leicht gelb gefärbte Lösung wurde stark eingeengt und mit dem achtfachen Volumen Alkohol versetzt. Die ausgeschiedene Krystallmasse wurde in wenig Wasser gelöst, mit Tierkohle entfärbt und nach dem Einengen Alkohol zugesetzt, bis eine schwache Trübung erfolgte. Nach einigen Stunden schieden sich die Krystalle in viereckigen Blättchen analysenrein aus. Ausbeute 13,7 g = 57%. Die Substanz schmilzt bei 190°–191°.

9,4824 mg Substanz verbrauchten 7,134 ccm n/100-H_2SO_4.
3,335 mg „ gaben 5,550 mg CO_2 und 2,375 mg H_2O.
3,515 mg „ „ 5,805 mg CO_2 und 2,575 mg H_2O.

Für $C_5H_{11}O_3N$.	Ber.:	C 45,11%	H 8,33%	N 10,52%
	Gef.:	45,39	7,97	10,53
		45,04	8,20	

Sie ist in Wasser leicht löslich. Die wässerige Lösung reagiert gegen Lackmus schwach sauer. Biuretreaktion ist schwach positiv.

III. γ-Methylguanido-β-oxy-buttersäure (V)
$NH_2.C(NH).N(CH_3).CH_2.CH(OH).CH_2.COOH$.

2 g γ-Methylamino-β-oxy-buttersäure wurden mit 1,9 g frisch dargestelltem Cyanamid in 20 ccm Wasser gelöst; nach Zugabe einiger Tropfen Ammoniak blieb die Lösung bei Zimmertemperatur stehen. Schon nach 2 Tagen trübt sie sich, den nächsten Morgen ist sie ganz durchwachsen von derben Krystallen. Diese Krystalle wurden abgesaugt und mit Alkohol bis zum Verschwinden des Dicyandiamids ausgekocht. Durch mehrfaches Umlösen des Rohproduktes in heissem Wasser und Einengen der Lösung wurde reine Methylguanido-β-oxy-buttersäure in einer Ausbeute von 1,12 g (= 42 % der Theorie) erhalten.

Zur Analyse wurde im Vacuum über P_2O_5 bei 100° getrocknet.

6,8395 mg Substanz verbrauchten 11,59 ccm n/100-H_2SO_4.
Für $C_6H_{13}O_3N_3$. Ber.: N 24.00 %
Gef.: 23,72

Sie krystallisiert in Prismen, schmilzt bei 201°–202°, in Wasser sehr leicht löslich. Die wässerige Lösung zeigt keine Sakaguchische Argininreaktion.

Zur Charakterisierung geeignet ist das salzsaure Salz. Zu seiner Darstellung wurden 0,2379 g γ-Methylguanido-β-oxy-buttersäure in 13,6 ccm n/10-HCl gelöst, die Lösung auf dem Wasserbad zur Trockne eingeengt, der Sirup erstarrte beim Erkalten zu einer strahlig-krystallinischen Masse; nachdem die letzten Spuren von Wasser im Vacuumexsiccator entfernt waren, wurde das Chlorhydrat in wenig absolutem Alkohol gelöst, filtriert, eingeengt bis zur Trockne und nochmals in Alkohol aufgenommen, die alkoholische Lösung mit trocknem Äther bis zur beginnenden Trübung versetzt. Nach einigen Stunden schied sich das Chlorhydrat in Prismen aus.

Zur Analyse wurde das Salz im Vacuumexsiccator getrocknet.

5,451 mg Substanz gaben 3,770 mg AgCl.
5,966 mg Substanz verbrauchten nach Kjeldahl 8,516 ccm n/100-H_2SO_4
6,864 mg Substanz „ „ 9,788 „

Für $C_6H_{13}N_3O_3 \cdot HCl$.	Ber.:	N 19,85%	Cl 16,74%
	Gef.:	19,98	17,10
		19,96	

Das Salz schmilzt bei 170°; es löst sich leicht in kaltem Wasser und Alkohol, gar nicht in Äther.

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CHANGEMENT CARACTÉRISTIQUE DE LA VITESSE DE LA SÉDIMENTATION DES HÉMATIES DANS LES SOLUTIONS DE CONCENTRATION OLIGOLYTIQUE.*

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INTRODUCTION.

Au cours de nos recherches sur le mécanisme de l'hémolyse au point de vue de la masse active mécanique, nous avons trouvé ce curieux phénomène que ce n'est pas dans la solution saline « iso-tonique », mais bien dans une solution de concentration beaucoup plus élevée que les globules rouges résistent le mieux (1935, 1936). Nous avons alors nommé provisoirement cette concentration caractéristique la « concentration oligolytique » en attendant de connaître à fond sa signification scientifique.

Depuis nous avons continué à étudier ce phénomène de concentration oligolytique des hématies à différents points de vue. En effet cette concentration nous a montré beaucoup de faits remarquables méconnus jusqu'ici. En étudiant l'influence du lavage des globules rouges à l'eau salée de concentration variée, nous avons démontré que le lavage des globules rouges à n'importe quelle concentration exerce toujours une influence soit positive soit négative sur la résistance globulaire et que l'individu dont les globules rouges montrent un plus haut degré d'hémolyse dans les solutions salines hypo-oligolytiques, montre, au contraire, un plus bas degré d'hémolyse dans les solutions salines hyper-oligolytiques que chez les autres individus et *vice versa*. Ce phénomène est comparable au mouvement de la bas-

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cule ou de la balance, dont le point d'appui forme la concentration oligolytique. Ce même phénomène a été, depuis, reconnu par plusieurs auteurs: Y. Egami (1936) dans ses études sur la différence individuelle bovine dans le degré de l'hémolyse a également trouvé le phénomène de la " bascule " selon l'individualité et que ce sont les substances dans le sérum effectives à l'abaissement du point de congélation qui déterminent ce phénomène. R. Ebina (1936) nous a révélé un fait analogue très intéressant en nous montrant que les globules rouges nucléés et non-nucléés diffèrent essentiellement dans leur hémolyse dans l'eau salée contenant une faible dose d'urée: l'hémolyse des globules rouges nucléés est ralentie par la présence de l'urée dans le milieu de concentration saline hypo-oligolytique et est accélérée, au contraire, dans le milieu de concentration saline hyper-oligolytique, tandis que chez les globules rouges non-nucléés c'est juste le contraire qui se produit. Ici aussi on trouve encore le phénomène de la « bascule ».

D'autre part le phénomène de la « bascule » a été reconnu maintes fois par divers auteurs dans leurs études sur l'influence qu'ont certaines substances hémolysantes sur l'hémolyse dans les milieux salins de concentrations variées: l'alcool éthylique par H. Hiraoka (1936), l'alcool méthylique par A. Asano (1936), la glycérine par I. Itinosawa (1936), les sels biliaires, la saponine, la digitonine, les savons etc. par S. Huzimoto (1936).

D'après Tchungchin Bak (1936) le pourcentage d'hémolyse dépend catégoriquement de la concentration saline du milieu, suivant qu'il soit hypo-oligolytique ou hyper-oligolytique; la concentration oligolytique forme une démarcation de ces deux caractéres tout à fait opposés. Z. Nisiyama (1936) en étudiant la signification de la concentration oligolytique au point de vue morphologique a constaté que les globules rouges prennent le diamètre le plus petit dans l'eau salée de concentration oligolytique et que le diamètre s'élargit selon que la concentration saline dans le milieu s'accroît ou diminue. En prenant en considération le changement de diamètre en trois dimensions des globules rouges suivant la concentration du milieu, il est arrivé à la conclusion que le volume même des globules rouges prend sa valeur la plus petite dans le milieu salin de concentration oligolytique.

N. Yakusizi (1936) en appliquant toutes les connaissances acquises jusqu'ici relatives à la concentration oligolytique des globules rouges, a réussi à trouver un moyen extraordinaire de séparer les nucléi des globules rouges nucléés. D'après ses résultats, c'est dans le milieu salin de concentration oligolytique que les nucléi sont séparés net par l'application d'un certain poison du protoplasma hémolysant et c'est aussi grâce à la concentration oligolytique que les nucléi isolés peuvent rester très longtemps en suspension homogène très stable. Grâce à sa découverte, la libération des nucléi par ce moyen peut se faire très tranquillement et précisément à l'œil nu sans recourir au microscope.

La concentration oligolytique, comme nous venons d'en énumérer les données expérimentales, nous fournit un tas de phénomènes affirmant l'importance de la concentration oligolytique et contredisant la notion classique «d'isotonie». Afin de connaître mieux ce phénomène singulier nous avons poussé nos observation un peu plus loin (1937) : la suspension d'hématies dans l'eau salée de concentration variée montre (1) une augmentation remarquable de viscosité à la concentration oligolytique, (2) un changement caractéristique dans l'abaissement du point de congélation de la suspension des hématies dans les solutions de concentration oligolytique vis-à-vis de celui du milieu et (3) un changement caractéristique dans la vitesse de la sédimentation des hématies dans des solutions salines de concentration oligolytique (1936). C'est ce dernier phénomène que nous décrivons dans ce présent mémoire.

PARTIE EXPÉRIMENTALE.

Depuis le travail de Fåhraeus en 1917 la vitesse de la sédimentation a été continuellement recherchée par un nombre considérable de chercheurs à presque tous les point de vue. Nous possédons aujourd'hui une bibliographie colossale comprenant largement le nombre de trois mille. La plupart de ces travaux ayant un but pratique, ne se rapportent qu'au sang des malades et il n'y a que relativement très peu de chercheurs qui se soient occupés des recherches scientifiques en vue de déterminer les facteurs influençant la vitesse de la sédimentation des globules rouges. Mêmes ces derniers, comme s'ils s'étaient donnés le mot, n'ont étudié le phénomène que dans le plasma et jamais dans le milieu simple et chimiquement bien déterminé. Nous trouvons à peine quelques travaux traitant la vitesse de la sédimentation dans le milieu simple, par exemple, R. Brinkman et F. H. Wastl (1921), L. Berczeller et F. H. Wastl (1924) l'ont étudié dans la solution de Ringer; K. Burker (1922), B. Behrens (1924) dans le liquide conservateur de Hayem, E. Block et Z. Oelsner (1923) dans l'eau salée physiologique. D'autre part, Friedmann (1925), Enocksson (1931) l'ont étudié dans le plasma additionné de chlorure de sodium et Demurtas (1932), Z. Uehara (1930) et K. Kura (1926) ont étudié l'influence exercée par l'addition de chlorure de calcium dans le plasma. Tous leurs résultats sont d'accord sur le fait de la retardation par la présence de ces sels minéraux, sauf Demurtas, dont les résultats montrent quelquefois une retardation et d'autrefois plutôt une accélération.

Notre but essentiel d'étudier la vitesse de la sédimentation des globules rouges consiste principalement à connaître de plus près la propriété physico-chimique du phénomène de la concentration oligolytique. Pour cela il nous a fallu tout d'abord établir une technique convenable pour étudier systématiquement l'influence de la concentration saline dans le milieu sur la vitesse de la sédimentation. Dans ce but, nous avons, dans notre mémoire précédent (1937), étudié systématiquement les conditions qui déterminent la vitesse de la sédimentation des globules rouges dans les systèmes simples et bien déterminés, savoir: les globules rouges de diverses espèces animales, la concentration des globules rouges dans la suspension, la masse active mécanique, la température, la concentration d'ion hydrogène, la gravité et la centrifugation, la hauteur (la longueur) et le diamètre du tube ainsi que le degré d'inclination du tube (1937) etc. Enfin nous avons formulé une technique adéquate en fixant constants les facteurs déterminant la vitesse de la sédimentation et nous avons étudié l'influence de la concentration saline sur elle.

1. *Tubes dans lesquels la sédimentation a été observée.*

Dans un travail précédent (1937) nous avons constaté que le *diamètre* du tube n'a pas d'influence sur la vitesse de la sédimentation, au moins dans la limite que nous avons examinée, de 7 cm. jusqu'à 4 mm de diamètre, et que la ***hauteur*** du tube non plus n'influence nullement la vitesse de la sédimentation, avant que « l'effet du fond » n'entre en jeu vers la fin de la sédimentation ; alors « l'effet du fond » commençant à apparaître retarde la vitesse de sédimentation.

Toutes ces circonstances mises en considération, nous nous sommes servis des tubes suivants: les tubes en verre à bout rond, de 8 mm. de diamètre et de 140 mm. de longueur, de grosseur homogène de bas en haut, choisis par calibration. Ils portent la graduation depuis le bas jusqu'à 7 cc. (112 mm. de hauteur) du haut, la partie au-dessus reste sans graduation et quant ils sont dressés debout en rang la graduation de 7 cc. forme une ligne droite c'est à dire est à la même hauteur pour tous les tubes.

2. *Globules rouges employés et leur concentration dans la suspension.*

La courbe de la concentration saline et de la vitesse de la sédimentation des globules rouges variant beaucoup, d'après nos propres expériences, suivant l'espèce animale (1937), nous avons pris, comme matériaux représentants, des globules rouges du sang défibriné de deux espèces animales, le bœuf et l'oie.

Le niveau des globules rouges que forment les globules rouges dans des suspensions de la solution saline au cours de leur sédimentation, marque quelquefois une délimitation très distincte, d'autres fois très vague et tellement dégradée qu'il n'est jamais possible de lire la distance de la sédimentation. Cela dépend de la proportion de la quantité du milieu et de la quantité d'hématies, autrement dit de la concentration des hématies. Nous avons choisi, d'après nos propres expériences, le rapport à raison de 1 cc. de purée d'hématies dans 6 cc. de solution saline.

3. *Technique pour la vitesse de la sédimentation globulaire.*

Prenant une série des tubes dont nous venons d'écrire, on met dans chacun une solution saline à étudier de concentration différente jusqu'à la graduation de 6 cc. (95 mm. de hauteur) en faisant attention de ne pas mouiller la paroi, on y ajoute 1 cc. de la purée d'hématies, puis on les bouche avec les bouchons en caoutchouc. Après quoi on délaye les globules plus ou moins collés à la paroi du tube, en les retournant légèrement plusieurs fois et en s'assurant que la suspensions est parfaitement homogène. Dans cette opération on a peu à se soucier de l'effet provoqué par la masse active mécanique inévitable, car la masse active mécanique qu'on donne préalablement à la suspension n'influence guère sur la vitesse ultérieure de la sédimentation Quand les globules rouges sont délayés en suspension homogène pour tous les tubes, on les retourne encore une fois tous ensemble pour mettre le point de départ de la submersion égal pour tous les tubes.

Ensuite la distance entre le niveau du liquide et le niveau que forme la colonne des globules rouges est mesurée à l'intervalle du temps déterminé. Une précaution toute spéciale à prendre dans la technique est, comme beaucoup d'expérimentateurs l'ont remarqué, de dresser les tubes absolument en position perpendiculaire, parce que même d'une très légère inclination résulte une accélération remarquable de las édimentation, surtout quand il s'agit de recherches, comme les nôtres, dans lesquelles on ne veut pas laisser échapper le moindre changement dans la vitesse de sédimentation.

4. *Solutions salines de concentrations variées.*

Dans ces expériences, nous avons étudié les sels suivants: chlorure de potassium, chlorure de sodium, bromure de potassium, bromure de sodium avec 2 molécules d'eau de cristallisation, chlorure de calcium avec 2 molécules d'eau, chlorure de strontium avec 6 molécules d'eau et chlorure de baryum avec 2 molécules d'eau.

La concentration saline des solutions a été désignée plutôt en molécules-grammes qu'en pour cent (ou en gr/dl) et dans cette désignation l'eau de cristallisation n'a pas été mise en compte. La sédimentation a été étudiée dans les solutions salines de concentrations variées suivantes, pour les globules rouges du bœuf: 0,12, 0,14, 0,15, 0,17, 0,20, 0,25, 0,30, 0,40, 0,50, 0,60, 0,70, 0,80, 0,90, 1,00 et 1,20 molécules-grammes et pour les globules rouges de l'oie on a ajouté à cette liste 0,10 et retiré 1,20.

5. *Ambiance et intervalle de temps d'observation.*

La température de l'ambiance dans laquelle nous avons effectué nos expériences était fixée à 20°C. La distance entre la surface du liquide et le niveau de la couche des globules rouges a été mesurée à l'intervalle convenable, mais pour les globules rouges du bœuf, il a été un peu plus espacé que pour les globules rouges de l'oie, car les derniers tombent plus vite que les premiers.

6. *Résultat des expériences.*

La vitesse de la sédimentation des globules rouges examinée dans les solutions salines de concentrations variées est résumée dans les tableaux ci-joints (I et II), dans lesquels la vitesse représente la distance en mm par heure, ou plus exactement la valeur de la tangente obtenue par les tracés graphiques du temps et de la distance entre la surface du liquide et le niveau de la couche d'hématies. Dans ces systèmes la chute des hématies est parfaitement uniforme si le milieu n'est pas trop concentré. La vitesse de la sédimentation qui n'est plus uniforme dans les solutions trop concentrées est marquée d'un astérique dans les tableaux.

D'autre part nous avons donné les tracés graphiques de la concentration et de la vitesse de la sédimentation dans les figures 1 à 12 ci-jointes. Dans ces tracés graphiques, les ordonnées représentent la distance de la chute des globules rouges et les abscisses la concentration saline de la solution. La direction des

Fig. 1. ClNa; bœuf.

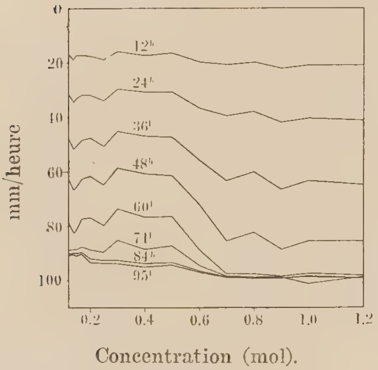


Fig. 2. ClK; bœuf.

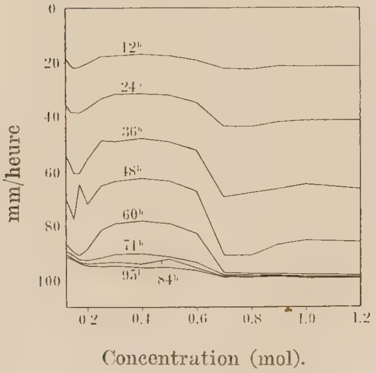


Fig. 3. BrNa; bœuf.

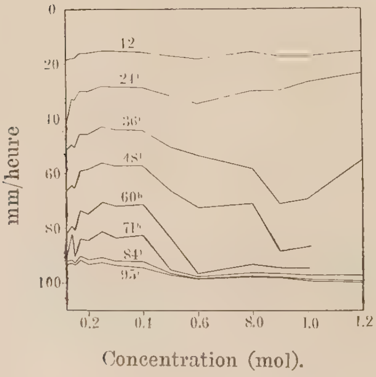


Fig. 4. BrK; bœuf.

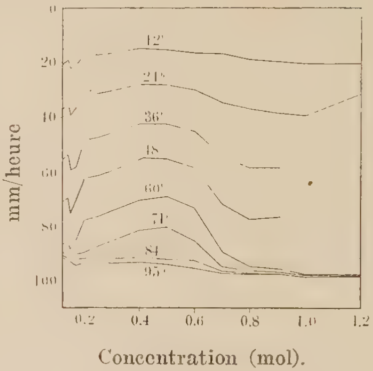


Fig. 5. Cl^2Ca; bœuf.

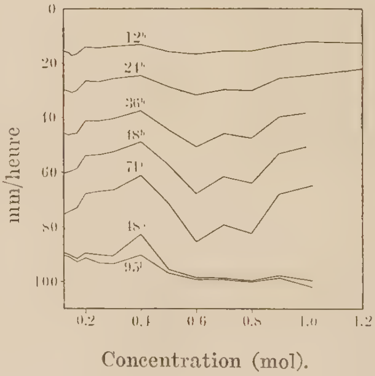


Fig. 6. Cl^2St; bœuf.

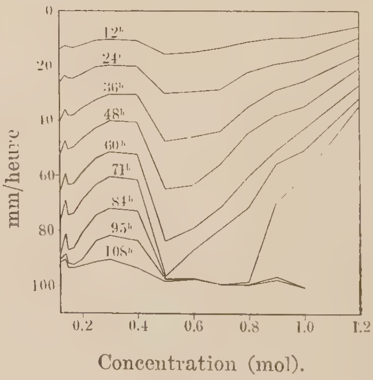
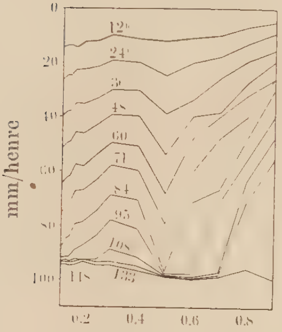
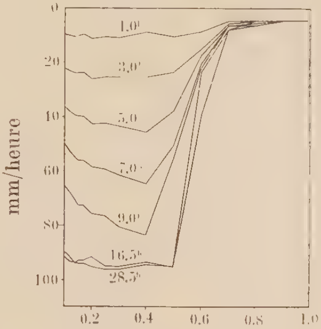


Fig. 7. Cl^2Ba; bœuf.



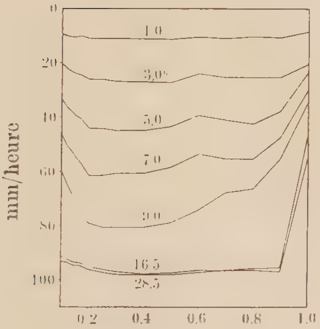
Concentration (mol).

Fig. 10. Cl^2Ca; oie.



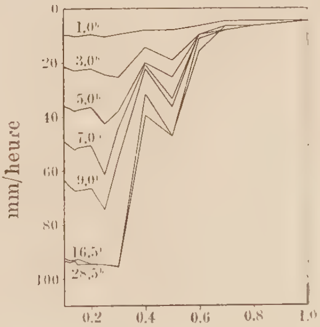
Concentration (mol).

Fig. 8. ClNa; oie.



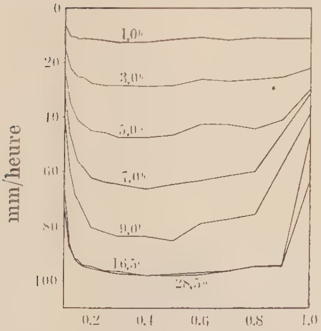
Concentration (mol).

Fig. 11. Cl^2St; oie.



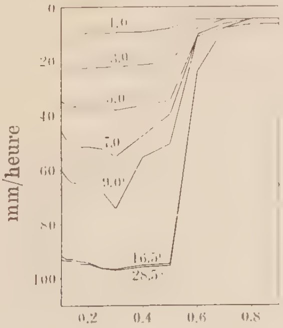
Concentration (mol`.

Fig. 9. ClK; oie.



Concentration (mol).

Fig. 12. Cl^2Ba; oie.



Concentration (mol).

ordonnées est prise exprès dans le sens contraire. C'est pour faciliter la compréhension du phénomène par l'apparence des courbes de façon que leur forme nous donne directement l'idée du volume du sédiment auquel nous sommes habitués. Dans une figure nous avons donné plusieurs courbes. Elles représentent de haut en bas, la marche du temps, c'est à dire que chaque courbe montre le changement de la vitesse de la sédimentation selon la concentration saline à un moment donné et la lettre *h*. signifie l'unité de temps heure.

D'après ces données numériques ainsi que les courbes, le fait le plus remarquable (avec globules rouges du sang de bœuf) est qu'on trouve une élévation très large dans les courbes suivant l'accroissement de la concentration. Ce phénomène est commun à tous les sels à cation mono-valent: ClNa, ClK, BrNa et BrK.

Cependant la forme et l'étendue de cette élévation varient plus ou moins selon le sel. Le chlorure de potassium et le bromure de potassium, par exemple, montrent une colline à base plus large et à sommet plus arrondi que celle du chlorure de sodium et du bromure de sodium. Tandis qu'avec les solutions des sels à cation di-valent la colline est beaucoup plus serrée à gauche qu'avec les solutions de sels à cation mono-valent. Entre les trois sels à cation di-valent que nous avons examinés, il y a une différence dans la forme de la colline selon le sel, le chlorure de strontium et le chlorure de baryum en montrant un caractère représentant et le chlorure de calcium le moins contrastant. Un caractère encore plus distinct et essentiel entre les sels à cation mono-valent et les sels à cation di-valent se trouve dans la forme des courbes après cette colline. Dans les solutions plus concentrées que celles dans lesquelles se manifestent cette élévation caractéristique de la colline, les sels à cation mono-valent n'influencent guère l'élévation de la courbe, tandis que les sels à cation di-valent se distinguent des précédents par une élévation très caractéristique suivant l'accroissement de leur concentration.

Maintenant nous allons envisager ce que nous venons de voir dans les tracés graphiques au point de vue de la vitesse de la sédimentation globulaire. Dans ce but nous avons tracé les graphiques en prenant comme ordonnées la distance de la submersion des globules rouges et comme abscisse le temps. La chute des globules rouges étant un mouvement uniforme, tous les points d'observations reposent, en effet, sur une ligne droite si le niveau de la colonne globulaire ne s'approche pas trop du fond. Après avoir donné une correction mathématique aux valeurs numériques, expérimentales, nous avons calculé les valeurs de la tangente de façon qu'elles représentent en même temps la vitesse de la sédimentation des globules rouges en mm. par heure (voir le tableau I).

TABLEAU I.

Vitesse de la sédimentation des hématies du bœuf dans les solutions de divers sels minéraux.

Sels.	ClNa	ClK	BrNa	BrK	$Cl^{2}Ca$	$Cl^{2}St$	$Cl^{2}Ba$
Concentration en mol.	Vitesse de la sédimentation en mm. par heure.						
0,12	1,25	1,38	1,33	1,38	1,22	1,05	1,02
0,14	1,39	1,48	1,26	1,41	1,21	0,95	1,01
0,15	1,32	1,36	1,30	1,28	1,17	1,03	0,97
0,17	1,25	1,44	1,19	1,32	1,16	1,02	0,90
0,20	1,24	1,44	1,18	1,26	1,10	0,96	0,94
0,25	1,27	1,32	1,17	1,23	1,10	0,88	0,87
0,30	1,21	1,31	1,18	1,19	1,09	0,86	0,83
0,40	1,23	1,27	1,13	1,15	0,98	0,86	0,82
0,50	1,24	1,27	1,39	1,11	1,15	1,38	1,15
0,60	1,45	1,33	1,51	1,19	1,46	1,34	0,84*
0,70	1,80	1,92	1,20	1,52	1,18	1,18	0,74*
0,80	1,73	1,90	1,52	1,63	1,39	1,02	0,60*
0,90	1,90	1,83	2,43	1,60	1,27	0,81	0,44*
1,00	1,79	1,81	2,22	1,32	0,82	0,69	—
1,20	1,78	1,79	1,31	1,66	—	0,43	—

Les données numériques du tableau I nous montrent que la phase de concentration dans laquelle se manifeste la vitesse de la sédimentation la plus lente pour chaque sel se place:

ClNa	à	0,3	molécules-grammes
ClK	à	0,4–0,5	,,
BrNa	à	0,4	,,
BrK	à	0,4–0,5	,,
$\mathrm{Cl^2Ca}$	à	0,4	,,
$\mathrm{Cl^2St}$	à	0,25–0,4	,,
$\mathrm{Cl^2Ba}$	à	0,3–0,4	,,

Ce que nous venons de voir jusqu'ici est ce qui se passe avec les globules rouges du sang de bœuf, mais quand il s'agit des globules rouges de l'oie, les choses sont toutes différentes.

La forme de la courbe de la distance de la chute globulaire et de la concentration saline du milieu (de fig. 8 à fig. 12) montre une allure tout à fait contraire de celle du bœuf. Les courbes

TABLEAU II.

Vitesse de la sédimentation des hématies de l'oie dans les solutions de divers sels minéraux.

Sels.	ClNa	ClK	$\mathrm{Cl^2Ca}$	$\mathrm{Cl^2St}$	$\mathrm{Cl^2Ba}$
Concentration en mol.	Vitesse de la sédimentation en mm. par heure.				
0,10	6,20	4,18	7,25	6,85	6,20
0,12	7,03	6,11	7,12	7,08	6,59
0,14	7,17	7,28	7,45	7,00	6,78
0,15	7,53	7,82	7,77	7,14	6,88
0,17	8,00	7,90	7,73	—	6,89
0,20	8,39	7,95	7,90	7,44	7,03
0,25	8,63	8,72	8,19	8,25	7,12
0,30	9,07	9,04	8,63	5,75	8,10
0,40	8,48	9,10	9,33	5,64*	5,99
0,50	7,98	9,12	6,84	4,27*	6,72*
0,60	7,18	8,51	1,99*	0,64*	1,20*
0,70	7,67	8,10	0	0	0
0,80	7,56	8,10	0	0	0
0,90	6,22	7,50	0	—	0
1,00	3,20*	3,67*	0	0	—

avec les globules rouges du sang de l'oie, au lieu de montrer une élévation à leur première moitié, montrent au contraire une descente très remarquable. Ce fossé des courbes est très large avec les solutions des sels à cation mono-valent, en s'étendant jusqu'à la concentration très avancée, tandis qu'avec les solutions des sels à cation di-valent (Cl^2Ca, Cl^2St, Cl^2Ba) le fossé est très serré vers la gauche, puis après ce fossé les courbes précipitamment remontent de nouveau.

La vitesse de la sédimentation des globules rouges du sang de l'oie est indiquée dans le tableau II.

La phase de concentration des solutions dans lesquelles se manifeste la vitesse la plus grande de la sédimentation globulaire pour chaque sel se place:

$ClNa$	à	0,3	molécules-grammes,
ClK	à	0,4–0,5	„
Cl^2Ca	à	0,3–0,4	„
Cl^2St	à	0,25	„
Cl^2Ba	à	0,3	„

Ainsi, dans les solutions des sels à cation mono-valent, la vitesse de la sédimentation la plus grande diminue graduellement suivant l'accroissement de la concentration, tandis que dans les solutions des sels à cation di-valent la diminution de la vitesse est brusque.

Nous sommes très étonnés de voir la concordance frappante qui existe entre la concentration des solutions salines dans sesquelles a lieu la vitesse de la sédimentation globulaire *la plus petite* avec les globules rouges du bœuf et la concentration des solutions salines dans lesquelles la vitesse de la sédimentation globulaire est *la plus grande* avec les globules rouges de l'oie.

Comme nous l'avons déjà remarqué plus haut, les globules rouges de l'oie montrent une diminution brusque dans leur vitesse maximum dans les solutions des sels di-valents suivant l'accroissement de la concentration, tandis que la diminution de la vitesse de la sédimentation des globules rouges du bœuf est beaucoup moins brusque. Par conséquent la comparaison des concentrations caractéristiques communes à ces deux espèces animales doit se faire plutôt à la limite inférieure de leur concentration. Alors on trouve effectivement une concordance assez satisfaisante entre elles.

Ce phénomène extraordinaire dû à une concentration particulière n'est au fond autre chose que le phénomène analogue de la concentration oligolytique que nous avons décrite maintes fois à propos de l'hémolyse.

Dans les solutions salines très concentrées la ligne de délimitation entre la colonne globulaire et le milieu surnageant devient souvent très vague suivant l'accroissement de la concentration saline et la chute des globules rouges ne décrit plus alors un mouvement uniforme. Ceci est vrai pour les solutions des sels à cation mono-valent ainsi que pour les solution des sels à cation di-valent et aussi pour les globules rouges du sang du boeuf et pour les globules rouges du sang de l'oie.

L'ambiguité du niveau de la colonne globulaire peut s'expliquer par le fait que, dans les solutions salines concentrées, les globules rouges se déformant et changeant leur volume ainsi que leur densité ne tombent pas tous à une vitesse égale et le niveau équivoque de la couche globulaire ne montre en somme que la vitesse de la sédimentation des mélanges des globules rouges de volume et de densité très variés.

Quand on compare la vitesse de la sédimentation des globules rouges (du bœuf et de l'oie) dans les solutions des divers sels minéraux de concentration équimoléculaire, concentration dans laquelle se manifeste le changement très caractéristique décrit plus haut, on trouve qu'elle est plus grande dans les solutions des sels à cation mono-valent que dans les solutions des sels à cation di-valent et que parmi les derniers sels, la vitesse de la sédimentation globulaire semble être inversement proportionelle à la grandeur du poids moléculaire des sels ou du poids atomique de leur cations, bien qu'il n'en soit pas toujours ainsi pour les sels à cation mono-valent.

De ce fait on peut se demander si la vitesse de la sédimentation ne dépendrait pas tout bonnement de la densité du milieu.

Pour en décider nous l'avons examiné sur les tracés graphiques de la vitesse de la sédimentation globulaire comme ordonnées et de la concentration exprimée en pour cent ou en gr/dl et non en molécules-grammes comme abscisses. On ne trouve cependant jamais la même vitesse de sédimentation globulaire à la même concentration en pour cent dans les solutions de différents sels, elle dépend catégoriquement de la nature du sel. La densité de la solution bien qu'elle puisse entrer en jeu jusqu'à une certaine mesure, ne peut jamais, à elle seule, expliquer suffisamment le phénomène. De même la viscosité du milieu ne peut non plus expliquer le phénomène que la densité.

Maintenant nous allons envisager l'affaire du côté des globules rouges. Tout le monde sait que les globules rouges s'altèrent morphologiquement dans une solution saline et que cette déformation dépend de la concentration de la solution saline. Le changement morphologique des globules rouges dans les solutions salines de différentes concentrations a été étudié très souvent par divers auteurs, mais récemment Z. Nisiyama a développé ses investigations très en détail, en évaluant le changement de diamètre des globules rouges du sang de plusieurs espèces animales en trois dimensions, dans les solutions de chlorure de sodium de concentrations extensivement variées dans les deux sens hypo-oligolytique et hyper-oligolytique. D'après ses résultats, les globules rouges de bœuf prennent leur volume le plus petit dans les solutions de concentrations oligolytiques et au fur et à mesure que la concentration s'éloigne dans les deux sens: plus diluée ou plus concentrée, les cellules se gonflent de plus en plus. Par conséquent les globules rouges dans les solutions salines de concentrations oligolytiques (en molécules-grammes), auxquelles correspondent nos concentrations caractristiques à la vitesse de la sédimentation, peuvent être retardés dans leur chute à cause de la diminution de leur volume, supposé que leur densité reste inaltérée indépendamment de la concentration du milieu. Mais cette hypothèse est peu commode pour expliquer le phénoméne, car, comme nous l'avons vu plus haut, dans les solutions salines de concentration oligolytique les globules rouges du sang de l'oie, contrairement aux globules rouges de bœuf, prennent leur vitesse de sédimentation la plus petite. Malgré cela, nous devons croire que les globules rouges du sang de l'oie prennent aussi leur volume le plus petit dans les solutions de concentration oligolytique, comme les globules rouges discoïdes de bœuf, à en juger d'après le diamètre en trois dimensions évalué par Z. Nisiyama.

Ainsi on voit que la diminution de volume seule ne pourra jamais donner une raison suffisante pour expliquer le phénomène. En réalité les circonstances sont beaucoup plus compliquées qu'on ne pense ; le volume, la densité et la forme des globules rouges changent et le degré de ce changement même varie selon l'individualité cellulaire.

Le phénomène que nous venons de décrire n'est point, sans aucun doute, un phénomène facilement expliquable par nos connaissances courantes d'aujourd'hui. Toutefois il nous offre un intérêt extrême pour les études physico-chimiques des hématies surtout quand nous le comparons avec un tas de phénomènes énumérés dans notre introduction, dûs à la concentration ologolytique par l'analogie frappante qui existe entre eux.

CONCLUSION.

1. A la concentration oligolytique, les hématies du bœuf montrent une élévation et les hématies de l'oi montrent une descente caractéristiques dans la courbe concentration-sédimentation.

2. Les concentrations auxquelles les hématies du bœuf montrent la vitesse la plus petite de sédimentation et les hématies de l'oi montrent la vitesse la plus grande sont identiques.

3. La stabilité des suspensions des hématies dépend de la valence des cations des sels dissouts dans le milieu.

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ON THE HYDROGEN PEROXIDE ELECTRODE.

By

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In the course of the potentiometric study on the behaviour of catalase action in this laboratory it was found that hydrogen peroxide gave a fairly stable potential to the blanc platinum electrode in the solution of definite pH and that the magnitude of this potential was independent to the concentration of peroxide in wide range. This fact led us to carry out the present work with the hope of applying this electrode for the practical purpose of determination of H-ion activity. The result is not so promising as we expected. But owing to theoretical interests we are going to publish some discussions on the behaviour of this electrode.

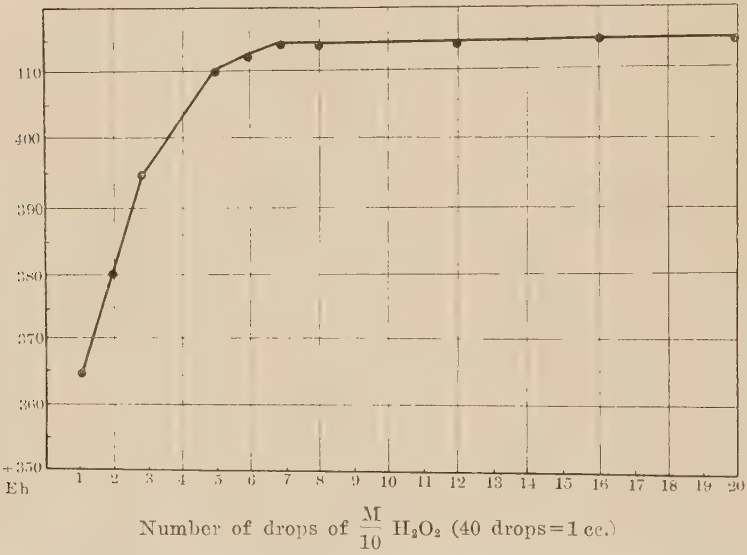
I. The method and the effect of H_2O_2-concentration on the magnitude of redox potential.

Into the vessel of about 30 cc. capacity was introduced 10 cc. of buffer into which the blanc platinum plate electrode (0.5 × 0.5 cm.) was dipped. As the reference electrode, the saturated calomel electrode was used. The connection between both electrode solutions was realised by KCl-agar bridge. The whole system was placed in the water thermostat kept at 20°C. The E. M. F. of the reference electrode was often controlled by referring to $M/10$ KCl-calomel-electrode. It's Eh value was found to be 0.2488 v. at 20°C. The potentiometer of the Shimazu Co., was used throughout this experiment, which enables the reading of 0.5 m. V. unit accurately and 0.1 m. V. with fair exactitude when we read the meniscus through a magnifying glass.

When the E. M. F. of the electrode became nearly steady one drop of $M/10$ H_2O_2 solution was run down from the burette under constant stirring with aeration of O_2-gas and the potential change was read minute by minute.

One of the typical examples is illustrated in Fig. 1.

Fig. The potential-concentration curve.



As will be seen from Figure I the potential sprung up abruptly at the beginning of the addition of H_2O_2 then gradually until almost constant at the further addition. The rise of the oxidations-potential is not so high as we met with other oxidation agents as potassium permanganate or bichromate solutions. This is a most typical behaviour of hydrogen-peroxide, which may be ascribed to dual nature as oxidant and reductant, as already depicted by Itoh (1935) in his study on the action of catalase.

II. The relations between pH of the solution and the redox potential.

Using acetate, phosphate, phthalate and borate buffers for respective hydrogen-ion concentrations, we measured the redox potential at the addition of hydrogen peroxide in the same manner as described in section 1, and recorded the highest value attained in Table I and the accompanying Figure II.

TABLE I.
Relation between pH and the potential value.

Sorts of buffer	pH measured by quinhydron-or H_2-electrode	Redox-potential in m. V.
1 *N*. H_2SO_4 solution	0.117	564.0
M/10 HCl solution	1.17	517.4
M/5 phthalate buffer	3.03	416.0
M/15 acetate buffer	3.62	384.0
”	3.82	375.0
”	4.23	383.7
”	4.46	341.5
”	4.64	329.0
M/15 phosphate buffer	5.04	309.0
”	5.98	277.0
”	6.97	209.0
M/10 boric acid-KCl-NaOH	8.04	167.0
buffer	8.43	140.0
”	8.78	125.0
”	9.20	unstable
”	9.60	”
”	10.00	”

As will be seen from the figure, the pH-potential curve is nearly straight in the acid range of the solution. In alkaline range the potential is not stable, perhaps owing to the rapid decomposition of hydrogen peroxide.

Since in the phosphate mixture of $pH=6.97$ the potential is 209,0 m. V. and in 1 *N* sulphuric acid of $pH=0.117$ the potential is 564.0 m. V. for one unit change in pH, there is a variation of 51.8 m. V. Hence the above straight line can be expressed by the equation

$$E=E_0-51.8\times pH.$$

By putting $E=564.0$ and $pH=0.117$ we get $E_0=570.0$, so that we can write the above equation as follow:

$$E = 570-51.8\times pH.$$

$$pH = \frac{570-E}{51.8}.$$

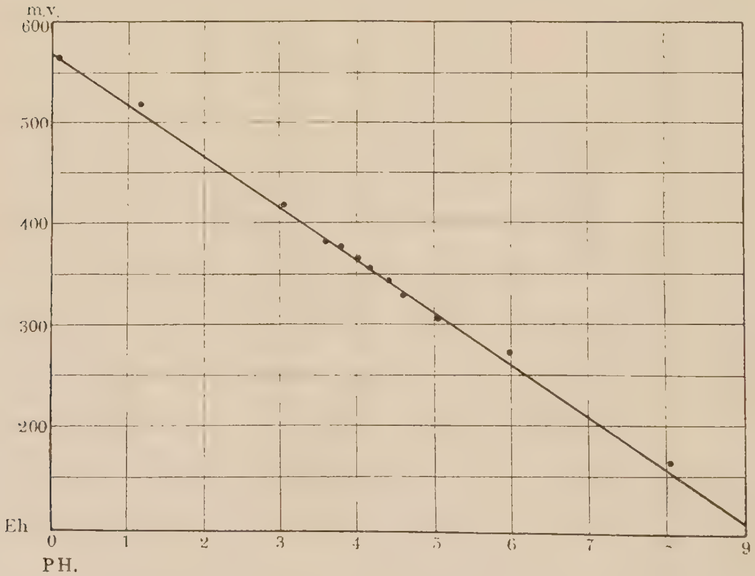
III. Theoretical discussions.

H_2O_2-electrode can be looked upon as oxygen-electrode. but it differs from O_2-electrode in the sense that it may be considered rather as atomic oxygen may react with platinum to form PtO_4 or PtO_3 and this state of platinum seems to reach to a saturated point in a relatively low concentrations of hydrogen-peroxide. because the maximum potential can be attained at a small concentration of hydrogen peroxide and is independent of the increase in its concentration.

At this equilibrium point atomic oxygen may react with hydroxyl ions in the following manner,

$$O + 2H_2O + 2e = 2OH^-.$$

Fig. II The potential-pH curve



Hence, the potential difference at the electrode surface can be represented as

$$E = E_0 - \frac{RT}{nF} \ln \frac{(OH^-)}{(O)^{\frac{1}{2}}},$$

$$E = E_0 - \frac{RT}{nF} \ln (OH^-) + \frac{RT}{nF} \ln (O)^{\frac{1}{2}}$$

If we assume that the concentration of atomic oxygen is constant as above mentioned this equation can be written as follows.

$$E = \dot{E}_0 - \frac{RT}{nF} \ln (OH^-),$$

or

$$E = \ddot{E}_0 - \frac{RT}{nF} \ln \frac{1}{(H^+)},$$

or

$$E = \ddot{E}_0 - \frac{RT}{nF} pH.$$

Putting the universal value for K, n, F, and T, we get at 20°C

$$E_{(m.V.)} = \ddot{E}_0 - 58{,}1 \times pH$$

From this equation we can see that for unit change of pH there is 58.1 m. V. variation of the potential. Since we have found experimentally 51.8 m. V. for this value, there exists a deviations of 7.3 m. V. from the theoretical value. This is too much for the experimental error. But since the constancy of the concentration and state of oxygen on the platinum surface is not so valid and the potential difference at the junction was neglected in the calculation a few millivolt deviation is admitted. Moreover, the use of the blanc metal electrode has been often encountered with unexplained difficulties. We found that using the same solution the potential was not always reproducible with different electrode, sometimes a few millivolt difference being resulted. This might be ascribed to the purity of the metal itself or to the surface nature of the electrode. But with the same electrode we can get almost a reproducible value.

O_2-electrode, which was studied by a number of workers, is still far from the practical use. This may due to the slowness of the reaction $O_2 \rightleftarrows 2O$, so that O_2-electrode is considered rather as irreversible (Richards 1928). The H_2O_2-electrode, however, may be considered as reversible and used for the measurement of OH-ions activity only in acid range more conveniently than O_2-electrode, if certain precautions are paid for the peculiarity of the blanc metal electrode used.

Since we have many reliable methods for such a measurement, as H_2-gas electrode or quinhydron electrode, we publish this paper simply from some theoretical interests on the oxygen electrode and claim no practical use.

SUMMARY.

Using the H_2O_2-electrode the relation between the potential (m. V.) and the activity of H-ion in various buffer mixtures at 20°C can be represented by the equation

$$E = E_0 - 51.8 \times \text{pH}.$$

and, some theoretical discussions for this equation are given.

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ÜBER DEN EINFLUSS DER NAHRUNGSFETTE AUF DIE EIGENSCHAFTEN DER PHOSPHATID-FETTSÄURE DES FETUS.

Von

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EINLEITUNG.

Es ist wohl bekannt, dass das Nahrungsfett auf die Eigenschaften des Körperfettes irgend eine Einfluss ausübt. Nach den Experimenten von Shioji soll das Leberphosphosphatid durch den Einfluss des Nahrungsfettes seine Jodzahl verändern. Sinclair gibt, an dass auch die Jodzahl des Phosphatides der Gewebe bei der Aufnahme der eine höhere Jodzahl besitzenden Fette zunimmt.

Nun habe ich folgende Experiment ausgeführt, um festzustellen, ob die Phosphatid-Fettsäure des Fetus durch die Eigenschaften des Nahrungsfettes beeinflusst wird.

VERSUCHSMATERIAL.

Die mir zur Verfügung gestellten Tiere sind reife weibliche Ratten mit einem Körpergewicht von über 120 g. Sie wurden vor dem Experiment mit einer bestimmten Nahrung über 20 Tage lang gefüttert. Um die Ratten genau zur Schwangerschaft zu bringen, habe ich täglich die gefärbten Ausstrichpräparate der Scheidensmegma mikroskopiert, welche täglich in bestimmter Zeit aufgenommen wurde, und nur die weiblichen Ratten im I. Stadium des Geschlechtszyklus, d. h. in der Vorbrunstzeit, mit den männlichen zusammengelegt. Am nächsten Morgen wurde die Scheidensmegma wieder mikroskopiert. Wurden Spermien darin nachgewiesen, so habe ich es als Anzeichen der ausgemachten Kopulation betrachtet und denselben Tag als I. Schwangerschaftstag bestimmt.

Die gewöhnliche Hauptnahrung der Versuchstiere besteht aus Reis, welchem Gemüse in geeigneter Menge zugefügt wurde. Bei den Hauptexperimenten wurde ein künstliches Futter folgender Zusammensetzung benutzt:

Stärke	68. g
Kasein	18. g
Versuchsfett	20. g
Salz (McCollum)	3. g
Oryzanin (Vitamin B)	3. g
Biosterin (Vitamin A u. D)	ein wenig.

Jede 112 g des genannten Gemisches wurden mit 70 ccm Wasser geknetet, für etwa 10 Minuten erhitzt, und nach der Abkühlung etwas Rettichsaft zugesetzt. Von dieser Nahrung wurden täglich 20 g dem einzelnen Tier verabreicht.

Für das Versuchsfett brauchte ich Leinöl (Jodzahl 187) als das die ungesättige Fettsäure reichlich enthaltende und Kokosnussöl (Jodzahl 10) als das dieselbe in kleiner Menge enthaltende Fett. Zu spezifischem Zwecke wurde noch die Elaidinsäure angewandt.

VERSUCHSMETHODE.

Dem vorher hergestellen Alkohol-Ätherextrakt der Organe wurde gesättigte alkoholische Chlorkadmiumlösung zugesetzt, worauf sich der Alkohol-Äther abdampfen liess. Der dabei gewonnene Rückstand wurde mit Aceton durchgemischt.

Das Gemisch wurde dann in eine Zentrifugenröhre gegossen und nach Abkühlung bis auf -10°C zentrifugiert, um die klare Oberschicht abzutrennen. Diese Acetonspülung wurde mehrmals wiederholt, die gesamten im Aceton unlöslichen Substanzen gesammelt und in wenig 3 % Salzsäure-Alkohollösung aufgelöst; dann mit gesättiger Natronlauge stark alkalisch gemacht und verseift. Von dem verseiften Material wurde dann die Phosphatid-Fettsäure durch das Kumagawa-Sutosche Fettbestimmungsverfahren getrennt.

Die Acetonlösung wurde abgedampft, der Rückstand in wenig Alkohol aufgelöst, die Lösung nach Zusatz der Natronlauge erhitzt und verseift und in warmem Wasser aufgelöst, dann in den Scheidentrichter gegossen. Der Lösung im Scheidentrichter wurde gleichmengiger Alkohol zugesetzt, um eine 50 % Alkohollösung herzustellen, zu welcher noch Petroleumäther kam, und das Ganze tüchtig geschüttelt.

Nach der Trennung des die unverseifbare Substanz enthaltenden Petroläthers liess der Alkohol sich abdampfen, dann wurde nach gewöhnlichem Verfahren die Fett-Fettsäure getrennt.

Die Jodzahl wurde mittels der Mikrobestimmungsmethode nach Wijs gemessen.

Experiment 1.

Zuerst habe ich den Fettstoff der mit einer gewöhnlichen Nahrung gefütterten Ratte untersucht. So habe ich bei den 5 Ratten, welche mit Reis und Gemüse 20–40 Tage lang gefüttert worden waren, die Jodzahl der Phosphatid-Fettsäure und der Fett-Fettsäure der Leber und des Muskels gemessen. Die Resultate sind aus Tabelle I zu ersehen. Wie Tabelle I zeigt, beträgt die durchschnittliche Jodzahl der Fett-Fettsäure bei der Leber 98.3 und die der Phosphatid-Fettsäure 157.7, während bei dem Muskel die der Fett-Fettsäure sich auf 80.3 und die der Phosphatid-Fettsäure auf 153.5 beläuft. Daraus kann man wohl

Tabelle I.

Tiernummer	Leber		Muskel	
	Fettsäure		Fettsäure	
	Fett	Phosphatid	Fett	Phosphatid
1	96.8	164.6	80.7	153.7
2	93.3	162.7	85.6	150.3
3	107.6	158.7	77.8	154.5
4	109.1	152.4	78.2	155.5
5	84.8	150.1	79.3	149.0
Mittel	98.3	157.7	80.3	152.6

schliessen, dass die Jodzahl der Phosphatid-Fettsäure sowohl bei der Leber als auch bei dem Muskel viel höher als diejenige der Fett-Fettsäure ist.

EXPERIMENT 2.

Die mit normaler Kost gefütterten Ratten, welche vorher geschwängert worden sind, wurden am 20.–21. Schwangerschaftstage durch Schlag getötet und die Fettsäure der Leber und des Muskels wurde sowohl bei den mütterlichen Ratten als auch den Feten gemessen. Wegen des geringeren Gehaltes der Fettsäure wurden die 4–6 Feten zusammen gleichzeitig analysiert.

TABELLE II.

Tiernummer	Mutterkörper				Fetus	
	Leber		Muskel			
	Fettsäure		Fettsäure		Fettsäure	
	Fett	Phosphatid	Fett	Phosphatid	Fett	Phosphatid
6	100.7	143.1	89.4	154.3	104.7	125.0
7	65.9	147.4	73.7	149.0	77.2	109.4
8	96.9	153.1	77.7	158.2	95.9	103.5
9	97.7	158.2	79.3	150.6	97.5	114.8
10	102.2	158.5	82.0	155.2	104.0	119.3
Mittel	92.7	152.0	80.4	153.5	95.8	114.4

Die Resultate sind in Tabelle II angegeben. Nach Tabelle II ist die Jodzahl der Fettsäure der Leber und des Muskels bei den mütterlichen Ratten gleich der des normalen Zustands, während in der Leber nur Phosphatid-Fettsäure ein wenig abnimmt.

Bei Feten beträgt die Jodzahl der Fett-Fettsäure durchschnittlich 95.8, ist also etwas höher als dieselbe bei den mütterlichen Ratten, während die Jodzahl der Phosphatid-Fettsäure, welche durchschnittlich 114.4 beträgt, viel niedriger als diejenige der Phosphatid-Fettsäure der Leber und des Muskels bei den mütterlichen Ratten ist.

EXPERIMENT 3.

Folgende Versuche wurden unternommen, um festzustellen, wie weit eine Fütterung mit Fettnahrung die Jodzahl der Körperfett-Fettsäure der Ratte beeinflusst. Die Ratten wurden in zwei Gruppen geteilt, die eine wurde mit Leinöldiät und die andere mit Kokosöldiät gefüttert; dann wurde bei beiden Gruppen je am 5., 10., 20., 25., und 45. Tage die Jodzahl der Fett-

TABELLE III.

Diät	Diätdauer (Tage)	Tiernummer	Leber		Muskel.	
			Jodzahl	Mittel	Jodzahl	Mittel
Leinöl	5	50 51	148.4 143.0	145.7	102.2 112.0	107.1
	10	52 53	160.1 167.6	163.8	115.7 100.2	108.0
	15	54 55	161.5 181.0	171.3	114.0 121.7	117.3
	20	56 57	167.0 174.8	170.9	118.9 123.5	121.2
	25	58 59	166.5 174.7	170.6	133.8 118.2	126.0
	45	60 61	175.2 170.8	173.0	139.1 134.9	137.0
Kokosnussöl	5	70 71	84.3 76.3	80.3	54.0 56.8	55.4
	10	72 73	56.4 62.1	59.2	53.8 52.4	53.1
	15	74 75	72.3 59.2	65.7	55.0 68.6	61.8
	20	76 77	59.3 69.3	64.3	59.9 51.7	55.8
	25	78 79	71.0 67.2	69.1	56.8 48.6	52.7
	45	80 81	58.7 80.3	69.5	67.6 51.0	59.3

Fettsäure der Leber und des Muskels gemessen. Die Resultate sind in Tabelle III angegeben.

Wie in Tabelle III gezeigt, ist die Jodzahl der Leberfett-Fettsäure, während der Fütterungszeit mit Normaldiät durchschnittlich 98.3, am 5. Fütterungstage bei Leinöldiät nimmt sie aber durchschnittlich bis auf 145.7 zu und bei Kokosöldiät nimmt sie dagegen bis auf 80.3 ab. Bei zehntägiger Fütterung mit Leinöldiät beträgt sie ebenfalls 163.8 und bei Kokosöldiät reduziert sie sich bis auf 58.2, also kann man eine deutliche Zu- oder Abnahme der Jodzahl bemerken. Nach der auffallenden Schwankung der Jodzahl in kürzeren Zeiten zeigt diese weiter am 45. Fütterungstage gar keine Schwankung mehr.

Bei der Muskelfett-Fettsäure verhält es sich ganz ebenso; die Jodzahl erfährt auch hier in den ersten 5–10 Fütterungstagen eine intensive Schwankung, die aber dann fast aufhört.

Aus obigen Resultaten erkennt man, dass durch die über 10 Tage lange Fütterung mit Versuchsdiät die Jodzahl der Fett-Fettsäure der Ratte sich verändern lassen kann; also in den folgenden Experimenten wurde die Fütterungszeit immer für 20 Tage bestimmt.

EXPERIMENT 4.

Bei den mit Leinöldiät 20 Tage lang gefütterten Ratten wurde die Jodzahl der Fettstoffe von Leber und Muskel unter-

TABELLE IV.

Tiernummer	Leber		Muskel	
	Fettsäure		Fettsäure	
	Fett	Phosphatid	Fett	Phosphatid
11	181.0	172.3	121.7	165.0
12	190.9	172.3	140.2	165.5
13	161.5	175.9	114.0	169.0
14	167.0	160.3	118.9	164.1
15	174.8	170.9	123.5	166.3
Mittel	175.0	170.3	123.6	165.9

sucht; die Resultate sind in Tabelle IV angegeben. Wie diese Tabelle zeigt, haben die Jodzahlen im Vergleich mit denjenigen bei Normaldiät bei der Leberfett-Fettsäure um 77, bei der Leberphosphatid-Fettsäure um 13, bei der Muskelfett-Fettsäure um 43 und bei der Muskelphosphatid-Fettsäure um 13 zugenommen.

Experiment 5.

Sofort nach der Begattung wurde die Ratte mit Leinöldiät gefüttert und am 20. Tage, nämlich an dem der Geburt vorangehenden Tage wurden die Jodzahlen der Fettstoffe sowohl des Mutterkörpers als auch des Fetus gemessen; die Resultate sind in Tabelle V angegeben. Beim Muttertiere hat die Jodzahl der Leberfett-Fettsäure im Vergleich mit derjenigen bei Normaldiät durchschnittlich um 70, die der Leberphosphatid-Fettsäure gleichfalls um 26, der Muskelfett-Fettsäure um 37 und der Muskelphosphatid-Fettsäure um 6 zugenommen. Vergleicht man die Jodzahl der verschiedenen Fettstoffe des Fetus mit derselben bei Normaldiät, so ergibt sich, dass die der Fett-Fettsäure um 34 und der Phosphatid-Fettsäure um 19 zugenommen hat.

Tabelle V.

Tiernummer	Mutterkörper				Fetus	
	Leber		Muskel			
	Fettsäure		Fettsäure		Fettsäure	
	Fett	Phosphatid	Fett	Phosphatid	Fett	Phosphatid
16	178.8	182.3	125.6	149.8	122.6	129.3
17	153.8	171.4	119.1	166.7	122.9	133.8
18	160.6	171.0	109.1	154.5	119.0	131.4
19	166.0	183.8	114.4	153.0	150.0	129.3
20	161.4	189.1	116.2	167.4	137.6	140.3
21	160.3	173.0	121.9	165.5	129.0	134.6
Mittel	163.5	178.4	117.7	159.5	130.2	133.1

Experiment 6.

Wenn man die Jodzahl der Fettstoffe bei den mit Kokosöldiät 20 Tage lang gefütterten Ratten erforscht, so kommt man zu den in Tabelle VI gebenen Resultaten. Danach verändert sich die Jodzahl der Phosphatid-Fettsäure nicht, wohl aber zeigt die der Leber- und Muskelfett-Fettsäure eine deutliche Verminderung.

TABELLE VI.

Tiernummer	Leber		Muskel	
	Fettsäure		Fettsäure	
	Fett	Phosphatid	Fett	Phosphatid
22	75.9	163.0	65.7	155.4
23	78.4	161.1	68.3	150.1
24	63.4	165.9	63.0	150.6
25	82.3	165.1	61.8	159.5
26	59.3	150.1	55.8	150.3
Mittel	71.9	161.0	62.9	153.2

EXPERIMENT 7.

Die Jodzahl der Fettstoffe von Mutterkörper und Feten wurde bei den mit Kokosöldiät 20 Tage lang gefütterten Schwangerschaftratten untersucht; die Ergebnisse sind in Tabelle VII

TABELLE VII.

Tiernummer	Mutterkörper				Fetus	
	Leber		Muskel			
	Fettsäure		Fettsäure		Fettsäure	
	Fett	Phosphatid	Fett	Phosphatid	Fett	Phosphatid
27	74.9	158.1	54.4	146.7	68.0	112.3
28	79.3	168.1	59.8	153.9	69.0	115.4
29	68.6	170.5	51.8	143.1	74.3	109.6
30	61.9	158.4	52.4	155.7	67.9	117.8
31	80.1	167.9	81.5	160.8	101.2	120.1
32	69.9	160.5	53.9	159.0	74.9	120.6
Mittel	72.5	163.9	59.0	153.2	75.9	116.0

angegeben. Danach hat die Jodzahl der Fett-Fettsäure sowohl der Leber sowie der Muskel des Mutterkörpers als auch der Feten im Vergleich zu der bei Normaldiät abgenommen, doch ist die Schwankung der Jodzahl bei der Phosphatid-Fettsäure nicht klar erkennbar.

Stellt man nun die obigen Resultate zusammen, so ergibt sich, dass, wie in Tabelle VIII (Normalzeit) und Tabelle IX (Schwangerschaftzeit) gezeigt, die Muskel- sowie Leberfett-Fettsäure des Mutterkörpers und die Fett-Fettsäure der Feten durch die Arten des Nahrungsfettes beeinflusst und ihre Jodzahlen sich verändern, und dass dabei die Phosphatid-Fettsäure des Mutterkörpers sowie der Feten in ganz gleicher Weise beeinflusst werden. Nämlich die Jodzahl nimmt bei Leinöldiät zu, während die Schwankung derselben bei Kokosöldiät nicht bedeutend ist.

TABELLE VIII.

Diet	Leber		Muskel	
	Fettsäure		Fettsäure	
	Fett	Phosphatid	Fett	Phosphatid
Normal	98.3	157.7	80.3	152.6
Leinöl	175.0	170.3	123.6	165.9
Kokosnussöl	71.9	161.0	62.9	153.2

TABELLE IX.

Diet	Mutterkörper				Fetus	
	Leber		Muskel			
	Fettsäure		Fettsäure		Fettsäure	
	Fett	Phosphatid	Fett	Phosphatid	Fett	Phosphatid
Normal	92.7	152.0	80.4	153.5	95.8	114.4
Leinöl	163.5	178.4	117.7	159.5	130.2	133.1
Kokosnussöl	72.5	163.9	59.0	153.2	75.9	116.0

EXPERIMENT 8.

Wie oben gesagt, kommt eine bedeutende Veränderung der

Jodzahl der Phosphatid-Fettsäure des Mutterkörpers sowie der Feten bei Kokosöldität nicht zustande. Dies beruht darauf, dass die Jodzahl des Phosphatides sogar dann nicht schwankt, wenn auch die gesättigte Fettsäure des Kokosöls sich an dem Aufbau des Phosphatides wirklich beiteiligen sollte.

Somit habe ich die Elaidinsäure, welche in ihren Eigenschaften der gesättigten Fettsäure ähnelt, ausgewählt, und folgende Versuche angestellt, um zu erkennen, ob die Elaidinsäure als eine an dem Aufbau des Phosphatides sich beteiligende Fettsäure benutzt werden kann oder nicht. Zu diesem Zweck habe ich die Ratte mit Elaidinsäurediät gefüttert, und nach einer Reihe von Tagen die Elaidinsäure in Fettstoffen der Gewebe bestimmt. Bei Bestimmung der Elaidinsäure aus Organen der Ratte wurde mittels der Bleisalz-Alkoholmethode die Fettsäure in feste und flüssige Fettsäure getrennt, dann wurde die Jodzahl der festen Fettsäure gemessen und die Menge der Elaidinsäure in dieser berechnet.

So wurden in erster Linie bei der mit Normaldiät gefütterten Ratte Menge und Jodzahl der aus Leber- und Muskelfettstoff-Fettsäure getrennten festen Fettsäure gemessen; auf diese Weise wurde die Menge der dieser beigemischten ungesättigten Fettsäure berechnet; die Resultate sind in Tabelle X (A u. B) angegeben. Wie aus dieser Tabelle ersichtlich ist, überschreitet die Jodzahl der getrennten festen Fettsäure niemals 5, also be-

TABELLE X. (A)

Tiernummer	Phosphatid-Fettsäure					
	Leber			Muskel		
	Feste Fettsäure		Ungesättigte Fettsäure (%)	Feste Fettsäure		Ungesättigte Fettsäure (%)
	Menge (%)	Jodzahl		Menge (%)	Jodzahl	
A1	37.5	3.5	1.5	31.9	2.5	0.9
A2	36.1	5.2	2.1	30.7	1.3	0.4
A3	34.6	3.6	1.4	29.8	3.9	1.3
Mittel	36.0	4.1	1.9	30.8	2.6	0.8

TABELLE X. (B)

Tiernummer	Fett-Fettsäure					
	Leber			Muskel		
	Feste Fettsäure		Ungesättigte Fettsäure (%)	Feste Fettsäure		Ungesättigte Fettsäure (%)
	Menge (%)	Jodzahl		Menge (%)	Jodzahl	
A1	10.0	2.5	0.3	20.7	4.5	0.8
A2	18.1	6.5	1.3	26.0	5.0	1.4
A3	21.8	2.6	0.6	27.7	3.1	1.0
Mittel	16.6	3.9	0.7	24.8	4.2	1.1

trägt die Menge der an der festen Fettsäure begleitenden ungesättigten Fettsäure gegenüber derselben der Fettstoff-Fettsäure niemals über 2,1%. Dann wurden die gleichen Versuche an der mit Normaldiät gefütterten Schwangerschaftratte angestellt und die in Tabelle XI (A. u. B) angegebenen Resultate gewonnen. Daraus ist ersichtlich dass es sich bei den Leber- und Muskel-Fettstoffen des Mutterkörpers ganz ebenso wie bei der Normalratte verhält. Bei dem Fetus beträgt die Jodzahl der festen Fettsäure beim Phosphatid durchschnittlich 6.7 und beim Fett 8.0, und die Menge der in der festen Fettsäure enthaltenden ungesättigten Fettsäure beträgt gegenüber jener der Phosphatid- sowie Fett-Fettsäure respektiv 2.3% und 1.5%.

TABELLE XI. (A)

Tiernummer	Phosphatid-Fettsäure								
	Leber			Muskel			Fetus		
	Feste Fettsäure		Ungesättigte Fettsäure (%)	Feste Fettsäure		Ungesättigte Fettsäure (%)	Feste Fettsäure		Ungesättigte Fettsäure (%)
	Menge (%)	Jodzahl		Menge (%)	Jodzahl		Menge (%)	Jodzahl	
A4	27.4	6.7	2.8	32.4	4.3	1.5	34.3	6.1	2.3
A5	36.5	3.6	1.5	23.0	7.3	1.9	30.7	6.0	2.0
A6	36.5	4.4	1.8	20.5	6.3	1.4	30.0	8.0	2.7
Mittel	36.8	4.9	2.0	25.3	6.0	1.6	31.7	6.7	2.3

TABELLE XI. (B)

Tiernummer	Fett-Fettsäure								
	Leber			Muskel			Fetus		
	Feste Fettsäure		Ungesättigte Fettsäure (%)	Feste Fettsäure		Ungesättigte Fettsäure (%)	Feste Fettsäure		Ungesättigte Fettsäure (%)
	Menge (%)	Jodzahl		Menge (%)	Jodzahl		Menge (%)	Jodzahl	
A4	11.8	5.0	0.7	32.2	4.5	1.6	23.1	7.4	1.9
A5	28.3	3.0	0.9	34.5	2.9	1.1	15.3	8.7	1.5
A6	27.2	3.5	1.1	28.1	3.0	0.9	12.4	7.8	1.1
Mittel	22.4	3.8	0.9	31.6	3.5	1.2	16.9	8.0	1.5

EXPERIMENT 9.

Wenn wir an den Fettstoffen der Ratte, welche für 20 Tage mit dem um 20% Elaidinsäure enthaltenden Grunddiät gefüttert worden ist, die gleichen Versuche wie die oben erwähnten anstellen, so werden die in Tabelle XII (A u. B) angegebenen Resultate gewonnen. Daraus kann man ersehen, dass die Jodzahlen der festen Fettsäure alle bedeutend zugenommen haben, indem sie je beim Leberphosphatid durchsnittlich 50.6, beim Muskelphosphatid 34.4, beim Leberfett 46.4 und beim Muskelfett 42.7 beträgt. Wenn die Menge der Elaidinsäure berechnet wird, so beläuft sie sich beim Leberphosphatid auf 20.7% und beim

TABELLE XII (A)

Tiernummer	Phosphatid-Fettsäure					
	Leber			Muskel		
	Feste Fettsäure		Elaidinsäure (%)	Feste Fettsäure		Elaidinsäure (%)
	Menge (%)	Jodzahl		Menge (%)	Jokzahl	
A7	37.0	55.6	22.9	28.7	43.2	13.8
A8	36.0	40.6	16.2	25.5	31.0	8.8
A9	37.2	55.8	23.1	27.3	28.6	8.7
Mittel	36.7	50.6	20.7	27.2	34.3	10.4

TABELLE XII. (B)

Tiernummer	Fett-Fettsäure					
	Leber			Muskel		
	Feste Fettsäure		Elaidinsäure (%)	Feste Fettsäure		Elaidinsäure (%)
	Menge (%)	Jodzahl		Menge (%)	Jodzahl	
A7	8.3	59.9	5.5	34.3	48.0	18.3
A8	22.8	40.4	10.3	38.5	46.5	19.9
A9	9.7	38.8	4.2	30.4	33.5	11.3
Mittel	13.6	46.4	6.7	34.4	42.7	16.5

Muskelphosphatid auf 10.4%.

Die gleichen Versuche wurden dann bei der mit Elaidinsäurediät für 20 Tage gefütterten Schwangerschaftratte ausgeführt; die Resultate sind in Tabelle XIII (A u. B) angegeben. Demnach ist die Elaidinsäure beim Leberphosphatid des Mutterkörpers zu 14.8%, beim Muskelphosphatid zu 8.0%, beim Leberfett zu 9.7% und beim Muskelfett zu 14.6% enthalten. Beim Fetus nimmt die Jodzahl der festen Fettsäure auffällig zu, indem die des Phosphatides 20.1 und die des Fettes 21.3 beträgt; die Menge der Elaidinsäure sowohl in der Phosphatid-Fettsäure als auch Fett-Fettsäure zeigt respektiv 5.8% und 4.7%.

Aus den obigen Experimenten geht deutlich hervor, dass

TABELLE XIII. (A)

Tiernummer	Phosphatid-Fettsäure								
	Leber			Muskel			Fetus		
	Feste Fettsäure		Elaidinsäure (%)	Feste Fettsäure		Elaidinsäure (%)	Feste Fettsäure		Elaidinsäure (%)
	Menge (%)	Jodzahl		Menge (%)	Jodzahl		Menge (%)	Jodzahl	
A16	45.5	17.0	8.6	25.5	24.9	7.1	28.2	10.8	3.4
A11	35.2	43.9	17.2	21.8	25.9	6.3	24.3	23.5	6.3
A12	39.9	42.0	18.6	38.9	33.5	10.6	26.6	25.9	7.7
Mittel	40.2	34.3	14.8	28.7	27.4	8.0	26.4	20.1	5.8

Tabelle XIII. (B)

Tiernummer	Fett-Fettsäure								
	Leber			Muskel			Fetus		
	Feste Fettsäure		Elaidinsäure (%)	Feste Fettsäure		Elaidinsäure (%)	Feste Fettsäure		Elaidinsäure (%)
	Menge (%)	Jodzahl		Menge (%)	Jodzahl		Menge (%)	Jodzahl	
A10	14.3	41.7	6.6	34.8	35.8	13.9	10.0	13.3	1.5
A11	19.6	54.3	11.8	34.8	40.3	15.6	15.9	21.0	3.7
A12	20.7	47.2	10.8	31.7	40.5	14.3	26.7	29.7	8.8
Mittel	18.2	47.7	9.7	33.8	38.9	14.6	17.5	21.3	4.7

bei der Ratte die mit der Nahrung dargereichte Elaidinsäure nicht nur als Fettsäure an dem Aufbau des Phosphatids sowie Fettes von Leber und Muskel des Mutterkörpers Anteil nimmt, sondern auch unverändert die Plazenta durchziehend ebenso als Fettsäure zum Aufbau des Phosphatides sowie Fettes des Fetus angewandt wird.

Aus der Tatsache, dass die Elaidinsäure, welche in ihren Eigenschaften der gesättigten Fettsäure sehr nahesteht, sich auf diese Weise am Aufbau des Phosphatides und Fettes von der Ratte beteiligen kann, kann man wohl schliessen, dass die gesättigte Fettsäure in der Nahrung gleichfalls auch zum Aufbau des Phosphatides und Fettes angewandt werden kann.

ZUSAMMENFASSUNG.

1) Wenn man die Ratte mit Leinöl füttert, so steigt die Jodzahl der Fett-Fettsäure sowohl des Mutterkörpers als auch des Fetus, während sie bei Kokosöl sinkt.

2) Bei der ebenfalls mit Leinöl gefütterten Ratte ist die Jodzahl der Phosphatid-Fettsäure sowohl des Mutterkörpers als auch des Fetus hoch, aber bei der Kokosölfütterung ist die Veränderung der betreffenden Jodzahl nicht deutlich.

3) Wenn man die Ratte mit Elaidinsäurediät füttert, so beteiligt sich die genannte Säure am Aufbau der Phosphatide sowohl des Mutterkörpers als auch des Fetus.

SCHLUSS.

Aus obigen Tatsachen geschlossen nimmt die Fettsäure im Nahrungsfett die Plazenta unverändert passierend an der Zusammensetzung des Phosphatides des Fetus Anteil.

Zum Schluss spreche ich Herrn Prof. Dr. Y. Sueyoshi für seine Anregung und Anleitung zu dieser Arbeit und Herrn Prof. Dr. K. Ando für die liebenswürdige Unterstützung meinen besten Dank aus.

LITERATUR.

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ON THE ANTIGENIC SUBSTANCE CONTAMINATED WITHIN PHOSPHOLIPIN.

By

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I. Introduction.

It was reported from this laboratory by Kadzuo Kimidzuka (1933) that the pure lecithin prepared by the cadmium method has neither the property of the antibody inducer, nor that of the antibody detector, and also that the hydrogenated crude phospholipin can be separated into the chloroform soluble fraction which shows no antigenic character and the water soluble fraction which has an antibody detecting character. Owing to the lack of substance the antigen inducing property of this water soluble fraction was not tested by Kimidzuka. I have therefore untertaken to get a large amount of this water-soluble fraction and to test its antibody inducing character. The chemical nature of this substance was also studied in this experiment.

II. The immunisation with a crude lecithin.

1. *The preparation of crude lecithin.*

The yolks of 600 fresh eggs were separated from the white, passed through a fine linen gauze, added with 2 times its weight of pure aceton, thoroughly shaken and put into the ice chest until next morning. After filtration, the precipitate was dried over a water bath to free it from aceton. To this dry powder about 2–3 times its weight pure alcohol were added and well shaken for several hours. After the stay in the ice chest the extract was filtered clearly and condensed in vacuum at a temperature not exceeding 40° to a syrupy consistency under a slow current of carbon dioxide. The residue was dissolved into a very small amount of ether and precipitated with the additon of an excess of aceton. The solution with ether and the precipitation with aceton were repeated three times. The preparation thus obtained was called "crude lecithin" and preserved under aceton in a brown coloured bottle in an ice chest.

2. *Immunisation with the crude lecithin.*

Albino rabbits weighing about 2.5 Kilo-grams and showing no complement fixation reaction were used throughout this study for the immunisation experiments.

3 cc. of 10 per cent crude lecithin alcoholic solution were added to 30 cc. of 5 per cent solution of pig serum in physiological saline solution, and well mixed immediately. To keep the lipoid emulsion better sodium oleate was added also in proportion of 0.5 per cent. After the lapse of one hour 1 cc. of the mixture was injected into the ear vein for a week, and after an intermission of five days, injections were repeated again for a week. The procedure was repeated again, so that 21 injections were made in all. 5–7 days after the last injection, the blood was taken out and tested by complement fixation reaction.

3. *The complement fixation reaction.*

The complement fixation reaction was performed by the Browning Method. The antiserum was inactivated by heating it at 56° for 30 minutes, and diluted 10 times with physiological saline solution. As the antibody detector dilute lecithin solution was made by diluting 1 per cent alcoholic lecithin solution 30 times with physiological saline solution. Guinea pig serum was used always as the complement, and the amount of it, which was sufficient to hemolyse 0.5 cc. of 3 per cent ox red corpuscle suspension in the presence of 5 units of hemolysin, was taken as one unit. The hemolytic system was composed of 1 cc. of 3 per cent ox red corpuscle suspension, made of physiological saline solution, and 5 units of hemolysin.

In a series of test tubes, 0.5 cc. of antibody detector, 0.5 cc. of 10 per cent antiserum, and increasing amounts of complement as 2, 4, 6, 8 units were put in and well mixed. After allowing for 1.5 hours at 37°, with agitation at intervals 0.5 cc. of the sensitized corpuscle suspension were added and the tubes were put in the incubator at 37° for 1.25 hours, to test the degree of hemolysis at the end of that time.

The result of this experiment is shown in Table I.

Table I.

Complement fixation reaction.
Antibody inducer: crude lecithin.
Antibody detector: crude lecithin.

Rabbit No.	Unit of complement				Antibody detector + complement	Blood serum + complement
	2	4	6	8		
212	−	−	⧺	⧻	⧻	⧻
213	−	−	+	⧻	⧻	⧻
214	−	−	⧺	⧻	⧻	⧻

As can be seen from the table crude lecithin can induce the antibody production within the rabbit's body when repeatedly injected with pig serum, and the antibody thus obtained combines with crude lecithin, as the latter is used as an antibody detector.

III. THE ANTIGENIC CHARACTER OF HYDROGENATED LECITHIN.

1. *The hydrogenation of lecithin.*

About 2 gm. of platinum black were suspended in 150 cc. of 2 per cent alcoholic lecithin solution and the mixture was saturated at 60–70° with hydrogen gas, which was purified by bubbling through saturated mercuric chloride solution, 2 per cent potassium permanganate solution and 5 per cent sodium hydroxide solution in succession. After the disappearence of the yellowish colour of the alcoholic solution, which occurred usually when the current of hydrogen was passed for 20–30 minutes, the hydrogen gas was bubbled through one hour more, and then the mixture was left for the sedimentation of platinum black at 60–70° for 30 minutes. The filterate freed from platinum black was colourless and the white precipitate of hydro-lecithin separated out as cooling proceded. The supernatant liquid was hydrogenated again as above, and the filtrate of the second hydrolecithin precipitate was evaporated in vacuum, mixed with hydrolecithin precipitated formerly, separated, and powdered dry. This preparation was called "crude hydrogenated lecithin". Its N:P ratio was 1.49 and the iodine number was found as 3.3.

2. *The immunisation test with crude hydrogenated lecithin.*

5 cc. of hot 10 per cent solution of crude hydrogenated lecithin were put into 40 cc. of 5 per cent glucose solution containing sodium oleate in an amount of 0.5 per cent. After cooling 2.5 cc. of pig serum were added, and filled to 50 cc. with 5 per cent glucose solution. The mixture was put aside in an ordinary temperature for an hour, and 10 cc. of it were injected into each rabbit in the same manner as in the case of immunisation with crude lecithin.

3. *Browning's complement fixation test with antiserum obtained against crude hydrogenated lecithin.*

Antiserum obtained by the immunisation for crude hydrogenated lecithin was tested by the Browning complement fixation test with crude hydrogenated lecithin as an antibody detector. The latter was made by mixing 1 cc. of 1 per cent hot alcoholic crude lecithin solution with 30 cc. of hot distilled water, and 0.5 cc. was used after cooling. Directly prior to the addition of sensitized red corpuscles it was added with 0.5 cc. of 1.8 per cent saline solution.

The result of the test is given in Table II.

Table II.

Browning's test with crude hydrogenated lecithin antiserum and crude hydrogenated lecithin as detector.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
215	−	−	+	+++	+++	+++
216	−	−	−	+++	+++	+++
217	−	−	−	+++	+++	+++

As the table shows the antibody inducing character of crude lecithin is not destroyed by hydrogenation. It is rather augmented, perhaps by the change of its physical character as an antibody detector.

IV. THE ISOLATION OF THE WATER SOLUBLE FRACTION OF HYDROGENATED CRUDE LECITHIN.

The hydrogenated crude lecithin obtained as above was dissolved in chloroform and after the addition of a small amount of alcohol was extracted with water at pH=2 several times. The chloroform fraction was evaporated dry in vacuum at a temperature not exceeding 40° and recrystalized 3 times with acetic ether. The water fraction was washed with chloroform many times, neutralized and evaporated dry in vacuum. The residue was thoroughly extracted again by ether and chloroform. This water soluble, chloroform insoluble fraction was a light yellow powder, and weighed about 8.2 gm. It showed Hopkins-Cole's, Millon's, Biuret and lead sulphide reaction. Ninhydrin reaction was negative. It showed also precipitation with sulphosalicylic acid and trichloracetic acid. With picric acid a slight turbidity was observed. Molish's carbohydrate reaction was negative. Lecithin and cholin were absent. The analytical data obtained were as follows:

Ash	56.3 %
Total nitrogen	6.31
Amino-N	4.06
Phosphorus	0.0082
Sulphur	0.17

When the substance was dissolved in a small amount of water and separated by the addition of trichloracetic acid, the nonprecipitated portion contained 0.5 per cent total nitrogen and 0.348 per cent aminonitrogen. Phosphorus and sulphur were almost entirely precipitated by trichloracetic acid.

V. THE ANTIGENIC PROPERTY OF THE WATER SOLUBLE FRACTION OF CRUDE HYDROGENATED LECITHIN.

With the water soluble fraction isolated as described in the preceding section the immunisation test was performed. Rabbits were injected either with 2 cc. of its 1 per cent solution, made of physiological salt solution, or with 5 cc. of a mixture, which consisted of 4 cc. of its 2 per cent (saline) solution and 16 cc. of 10 per cent pig serum allowed to stand for one hour at ordinary temperature. The method of immunisation remained the same as in the case of impure lecithin. The Browning complement fixation tests were performed with this immum serum. The water soluble portion of hydrogenated impure lecithin, impure lecithin and hydrogenated impure lecithin were used as antibody dectectors. The water soluble portion of hydrogenated impure lecithin was also used as an antibody detector for antiserum obtained against impure lecithin or hydrogenated impure lecithin. The results are shown in Table III.

TABLE III.

Complement fixation test with water soluble portion of hydrogenated impure lecithin either as antibody inducer or as antibody detector.

1. Antibody inducer: Water soluble portion of hydrogenated impure lecithin.
Antibody detector: The same.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
202	−	−	+	+++	+++	+++
203	−	+	++	+++	+++	+++
204	−	−	+	+++	+++	+++

2. Inducer: Water soluble portion of hydrogenated impure lecithin + Pig serum.
Detector: Water soluble portion.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
205	−	−	+++	+++	+++	+++
206	−	++	+++	+++	+++	+++
207	−	−	+++	+++	+++	+++

3. Inducer: Water soluble portion of hydrogenated impure lecithin.
Detector: Hydrogenated impure lecithin.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
202	−	−	⧻	⧻	⧻	⧻
203	−	+	⧻	⧻	⧻	⧻
204	−	+	⧻	⧻	⧻	⧻

4. Inducer: Impure lecithin.
Detector: Water soluble portion of hydrogenated impure lecithin.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
212	−	+	⧻	⧻	⧻	⧻
213	−	−	+	⧻	⧻	⧻
214	−	−	⧺	⧻	⧻	⧻

5. Inducer: Hydrogenated impure lecithin.
Detector: Water soluble portion of hydrated impure lecithin.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
215	−	+	⧺	⧻	⧻	⧻
216	−	−	⧺	⧻	⧻	⧻
217	−	−	⧻	⧻	⧻	⧻

As is clear from these experiments the water soluble portion of hydrogenated crude lecithin shows an entire character of antigen.

VI. THE IDENTITY OF IMMUNOLOGICAL PROPERTIES OF IMPURE LECITHIN AND ITS WATER SOLUBLE PORTION WITH OVOVITELLIN.

As we have seen the water soluble portion of impure lecithin has the protein nature; it seems quite possible that this might be due to a portion of ovovitellin which migrated into the ether and alcohol during the preparation of lecithin and contaminated the lecithin as an impurity.

To decide whether this possibility is really occuring or not I have prepared pure ovovitellin free from lecithin and compared the immunological properties between this pure ovovitellin and the water soluble portion of hydrogenated impure lecithin.

1. *The preparation of ovovitellin free from lecithin.*

Ovovitellin was prepared by the method of Levene and Alsberg (1901). Yolks, freed from egg white as completely as possible, were pushed through gauze and well mixed with an equal amount of 10 per cent sodium chloride solution and shaken thoroughly with about half its volume of ether. After placing in an ice chest for 24 hours, the ether layer was withdrwan and treated with a fresh portion of ether. This extraction with ether was repeated three times. The water layer was then added to 20 times its volume of water. The resultant precipitate was separated from water after several hours, well shaken with a large quantity of water and separated again from the water. The precipitate was then dissolved into 10 per cent NaCl solution, and the resulting solution was washed thoroughly with ether several times. The watery solution was filtered and into the filtrate a large amount of water was added. The precipitate obtained was freed from water by thorough centrifigation, dried by ventilation under 40° and well washed with ether. Ovovitellin thus obtained gave the following analytical result.

Total nitrogen	16.81 %
Phosphorus	1.22
Sulphur	0.6

The solution of this ovovitellin gave no precipitation either with cadmium chloride or iodine solution, indicating that it was entirely free from lecithin and choline.

2. *The immunological reaction of immun serum against ovovitellin with crude lecithin and water soluble portion of hydrogenated crude lecithin.*

Rabbits were immunised with this ovovitellin and immun serum obtained was tested against ovovitellin, crude lecithin and water soluble portion of hydrogenated crude lecithin.

TABLE IV.

Complement fixation test between ovovitellin antiserum and ovovitellin, crude lecithin or water soluble portion of hydrated crude lecithin.

1. Antibody producer: Ovovitellin.
Antibody detector: Ovovitellin.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
226	−	−	−	−	⧻	⧻
227	−	−	−	−	⧻	⧻
228	−	−	−	+	⧻	⧻

2. Antibody producer: Ovovitellin.
Antibody detector: Crude lecithin.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
226	−	−	−	⧺	⧻	⧻
227	−	−	−	⧻	⧻	⧻
228	−	−	+	⧻	⧻	⧻

3. Antibody producer: Ovovitellin.
Antibody detector: Hydrogenated crude lecithin.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
226	−	−	−	⧻	⧻	⧻
227	−	−	−	⧺	⧻	⧻
228	−	−	+	⧻	⧻	⧻

4. Antibody producer: Ovovitellin.
Antibody detector: Water soluble portion of hydrogenated crude lecithin.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
226	−	−	⧺	⧻	⧻	⧻
227	−	−	−	⧻	⧻	⧻
228	−	−	⧻	⧻	⧻	⧻

3. *Immunological reaction of immun serum obtained against crude lecithin or water soluble portion of hydrogenated crude lecithin against ovovitellin as antibody detector.*

I have also tested the effectiveness of ovovitellin, freed entirely from ether soluble substance, as the antibody detector against the immune serum obtained by repeated injections of crude lecithin or the water soluble portion of hydrogenated crude lecithine.

TABLE V.

Complement fixation test between crude lecithin antiserum and ovovitellin.

1. Antibody producer: Crude lecithin.
Antibody detector: Ovovitellin.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
212	+	⧺	⧻	⧻	⧻	⧻
213	—	—	+	⧻	⧻	⧻
214	—	—	⧻	⧻	⧻	⧻

2. Antibody producer: Water soluble portion of hydrogenated crude lecithin.
Antibody detector: Ovovitellin.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
202	—	+	⧺	⧻	⧻	⧻
203	—	+	⧺	⧻	⧻	⧻
204	—	⧻	⧺	⧻	⧻	⧻

The result of this section and that of the preceding section indicates to us that the proteic substance contained in the water soluble portion of hydrogenated crude lecithin is no other substance than ovovitelline, which is contaminated in lecithin in the course of its preparation.

VII. THE IMMUNOLOGICAL TEST BETWEEN CRUDE LECITHIN, PURE LECITHIN AND CASEINOGEN.

Pure lecithin was prepared by the method of Levene and West (1918). This pure lecithin was found entirely inactive as antibody an detector as is shown already by Kmidzuka. I have also prepared pure caseinogen and antiserum was obtained against this caseinogen. For this anticaseinogen serum water soluble portion of hydrogenated crude lecithin was found entirely inactive as an antibody detector.

TABLE VI.

Complement fixation test between water soluble portion of hydrogenated crude lecithin and pure lecithin or caseinogen.

1. Antibody producer: Water soluble portion of hydrogenated crude lecithin.
Antibody detector: Purified lecithin.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
202	⧻	⧻	⧻	⧻	⧻	⧻
203	⧻	⧻	⧻	⧻	⧻	⧻
204	⧻	⧻	⧻	⧻	⧻	⧻

2. Antibody producer: Caseinogen.
Antibody detector: Water soluble portion of hydrogenated crude lecithin.

Rabbit No.	Units of complement				Detector + complement	Serum + complement
	2	4	6	8		
220	⧻	⧻	⧻	⧻	⧻	⧻
221	⧻	⧻	⧻	⧻	⧻	⧻
222	⧻	⧻	⧻	⧻	⧻	⧻

These findings clearly show us that purified lecithin lacks the substance which serves as antibody detector against antiserum obtained by the water soluble portion of hydrogenated crude lecithin and also that the water soluble portion of hydrogenated crude lecithin acts specifically only to antiovovitellin serum as an antibody detector, while it is entirely inactive against anti-caseinogen serum.

VIII. THE CONCLUSION.

The above mentioned results clearly indicate the fact that the substance which enables crude lecithin or its hydrogenated product to act as an antibody producer is of protein nature and it contains at least ovovitellin as one of its immunologically active substances. The possibility of the presence of protein other than ovovitellin is not naturally excluded in these experiments.

SUMMARY.

1. Hydrogenated crude lecithin has an antigenic character.
2. Hydrogenated crude lecithin can be separated into the chloroform soluble portion and water soluble portion. The chloroform portion is entirely inactive as antigen, while the water soluble portion acts as antigen.
3. The water soluble portion contain proteic substance.
4. The water soluble proteic substance of crude lecithin is found to contain ovovitellin by immunological test.

I wish to express my most sincere thanks to Professor S. Kakiuchi for his kind advice and encouragement in carrying out this investigation.

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THE REACTION OF AMINO ACIDS, PEPTIDES AND RELATED SUBSTANCES WITH SUGARS. I.

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I. INTRODUCTION.

The chemical reactions of amino acids, peptides and proteins with sugars have been investigated under various conditions by several sorts of methods, and have become one of the fundamental problems of the day.

From the change of optical rotation in the lapse of time, it has been observed by Neuberg and his associates (1925, 1926, 1927) that fructose combines promptly with amino acids at pH 7, while Euler and his collaborators (1926) reported that glucose combines slowly but more abundantly with amino acids in alkaline reaction (pH 7, 8, 9). And Euler calculated from the degree of the freezing point depression the grade of the combination between them. Giedroyć, Cichoncka and Mystkowski (1935), Przylecki, Cichoncka and Rafolowska (1936) also observed the change of optical rotation, while Weber and Versmold (1931) determined the freezing point depression. Borsook, Wasteneys (1925), Euler, Brunius and Josephson (1926) and Lieben and Getreuer (1932) found that amino acids, peptides and proteins have the ability of reducing methylenblue in neutral, or slightly acidic reactions, when incubated with glucose at pH 7, 8, 8.5, and Lieben and Getreuer assumed that they form Schiff's base and then take up a water molecule at their double bond and play the role of hydrogen donator. By means of van Slyke's amino-N measuring method, Borsook and Wasteneys (l. c.) observed that, after 48 hours incubation at 37°C, of the solutions containing pepton and glucose, or glycocoll and glucose, there is a decrease of the amount of amino-N, and that its amount is more pronounced in a more alkaline medium, the pH of the solution changing from 6.3 to 8.4 in the former solution and from 7.8 to 11.0 in the latter. Using the same method, Waldschmidt-Leitz and Rauchalles (1928) pointed out that the combination between dipeptides and glucose was maximum at pH 8, and they concluded that this fact confirms the opinion that an active group of dipeptidase has the nature of aldehyde. In the case of polysaccharid the combination of amino acids or proteins with sugars is studied by means of the phenomena of "Koazervation" (Jong and his collaborators: 1929, 1930, 1932), and adsorption (Przylecki and his associates: 1932, 1933, 1934, 1935, 1936). Concerning the irreversible reactions which take place in a strong alkaline medium or at high temperature, Watanabe (1932) investigated this very thoroughly.

The results of the experiments so far referred to and others coincide in the point that amino acids combine with glucose or so more abundantly in the more alkaline medium, and this seems to be the very natural consequence of the Bjerrum's Zwitterion construction of amino acids (1923). But the result of Waldschmidt-Leitz and Rauchalles, stating that the combination of dipeptides with glucose has the maximum at pH 8 is very curious viewed from this conception and also from the fact that the method of formol titration is widely applied to the determination of amino-N of peptides. Therefore, it is a matter of interest and of importance to investigate more throughly the change of the amount of amino-N in the mixture of amino acids or, especially, peptides and sugar in the course of time. If the result of Waldschmidt-Leitz and Rauchalles is really asserted, our conception of the molecular, especially the electric, structure of amino acids and peptides is surely to be modified.

II. MATERIAL AND METHOD.

In 1 litre buffer solution containing 45 gm. of KH_2PO_4 for enzym study (Kahlbaum) and 95 gm. of citric acid for analyse (Kahlbaum), amino acid or peptide is dissolved in about 0.018 mol with sugar in about 0.9 mol. When amine is used in place of amino acid and peptide its concentration is made approximately 0.009 mol. Amino acids used are glycocoll (Merck) and hydrochloride of ε-aminocapronic acid prepared from cyclohexanon according to Wallach's method by Dr. Y. Mutô of this institute, to whom I am indebted to express sincere thanks. For peptide, Fränkel's glycylglycin and l-leucylglycylglycin are used, and for amine, methylamine (Merck) and isobutylamine (Schuchardt). Kahlbaum's glucose, Merck's fructose and galactose are used as sugar. The pH of the solutions are adjusted by the addition of 4n NaOH solution to about pH 7, 8, 9, and estimated by the "Toyo pH test paper", the accuracy of which is ±pH 0.1 or so. When heavy water is used 99.55% deuterium oxyde ($d_4^{20}=1.1049$) of Norsk Hydro-Elektrisk is adopted, diluted roughly to 50%.

These solutions containing either amino acid, peptide or amine and sugar at pH 7, 8, 9 are allowed to stand in stoppered Erlenmeyer's flasks for 1 or 2 days at ordinary temperature, during which, from time to time, 2 cc. of the mixture are transfered to van Slyke's apparatus and the amino-N is measured.

III. THE CHANGE IN THE AMOUNT OF AMINO-N WITH THE LAPSE OF TIME.

1. Glycocoll + glucose.

The whole results are shown in Table I and Fig. 1.

As is seen from the table and figure amino-N decreases the more the higher the pH value of the solution is, and the equilibrium is attained after 2 or 3 hours at pH 7 and 8, while at pH 9 it is attained only after more than 9 or 10 hours. For convenience sake I shall call this type of change of amino-N the "amino acid type". At pH 9.6, or probably a little greater than 9.6, the amount of amino-N measured from the 6th hour onwards differs, whether or not the content of the Erlenmeyer’s flask is shaken immediately before it is transfered to van Slyke’s apparatus. Perhaps this may be due to some decomposition reaction, but it remains unstudied. So far as the reaction is adjusted only to pH 9 no such decomposition seems to take place under such conditions.

These whole results coincide with those in the papers by previous authors referred to aboved and may be explained by the conception of Zwitterion of Bjerrum.

TABLE I.

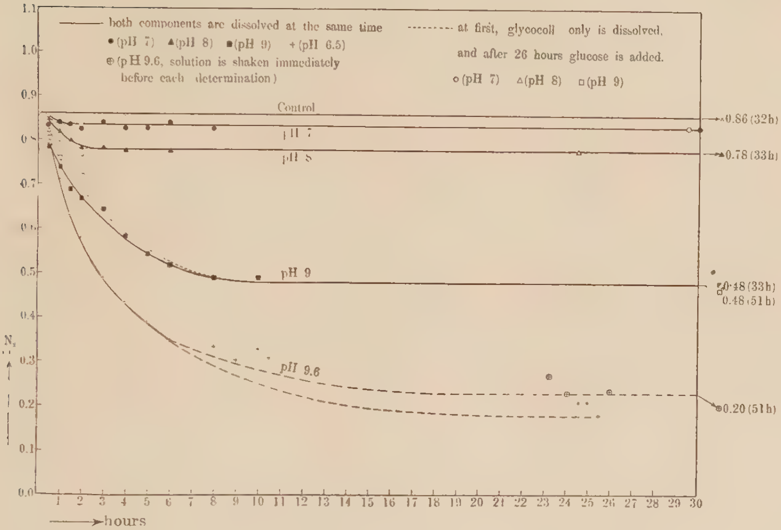
Glycocoll + glucose (0.018 mol : 0.9 mol)

Time (hours)	N_2 cc. (760 mm, 22°C)				
	pH 7	pH 8	pH 9	pH 9.6*	control**
0.2	—	—	—	—	0.86
0.5	0.835	0.85	0.785	0.79	—
1	0.84	0.82	0.74	0.71	—
1.5	0.835	0.80	0.69	0.63	—
2	0.825	0.785	0.67	0.58	—
3	0.84	0.785	0.645	0.49	—
4	0.83	0.78	0.585	0.435	0.855
5	0.83	—	0.545	0.39	—
6	0.84	0.78	0.52	0.35	—
8	0.83	—	0.49	0.335	—
9	—	—	—	0.305	—
10	—	—	0.49	0.33	—
30	0.835	—	—	—	—
32	—	—	—	—	0.86
33	—	0 78	0.48	—	—
51	—	—	—	0.20	—

* This pH value is not exact, the true value may be a little greater. From the 6 th hour onwards, the readings of the volume of N_2 gas are different whether or not the flask is shaken immediately before transference of samples of 2 cc. to van Slyke’s apparatus, and the readings of this region are not all given in Table I but in Fig. 1.

** Glycocoll only. pH is 9.

Fig. 1. Glycocoll + glucose.



2. Glycylglycin + glucose.

The results are given in Table II and Fig. 2.

The mode of curves are profoundly different from those of the previous series containing glycocoll and glucose, namely, while the change of the amount of amino-N is more accelerated at pH 8 than at pH 7, it is very retarded at pH 9. These results coincide exactly with those of **Waldschmidt-Leitz** and **Rauchalles**. After about 12 hours however the amount of change at pH 9 becomes larger than of that at pH 7, and after about 28 hours more than of that at pH 8. Therefore, after the lapse of about 28 hours, the effect of pH of the solution becomes the same as that by glycocoll. I shall call this type of curve the "dipeptide type."

In this case, at pH 7 and 8, the equilibrium was attained after several hours, while at pH 9 the equilibrium was not attained so far as the experiment continued.

3. l-leucylglycylglycin + glucose.

The results are recorded in Table III and Fig. 3.

Here the effect of pH is entirely reverse to that of the amino acid type until after the lapse of 5 or 6 hours. All differences between the amino acid type and the dipeptide type have become more manifest, and the change of amino-N at pH 9 remains smaller than of that at pH 7 even after 75 hours.

TABLE II.

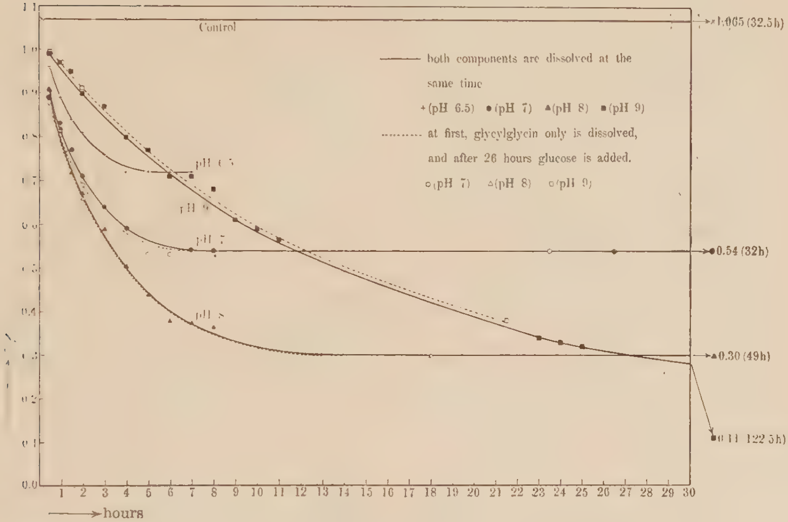
Glycylglycin + glucose (0.018 mol : 0.9 mol)

Time (hours)	N_2 cc. (760 mm, 22°C)					
	pH 6.5	pH 7	pH 8	pH 8.3*	pH 9	control**
0.2	—	—	—	—	—	1.07
0.5	0.96	0.89	0.91	0.915	0.99	—
1	0.89	0.83	0.82	0.805	0.97	—
1.5	0.84	0.77	0.72	—	0.95	—
2	0.81	0.71	0.67	0.67	0.90	—
3	0.76	0.64	0.59	—	0.87	—
4	0.72	0.59	0.50	0.505	0.80	1.07
5	0.72	0.53	0.44	—	0.77	—
6	0.72	0.53	0.38	0.38	0.71	—
7	0.72	0.54	0.375	—	0.71	—
8	—	0.54	0.365	—	0.68	—
9	—	—	—	—	0.61	—
10	—	—	—	—	0.59	—
11	—	—	—	—	0.56	—
23	—	—	—	—	0.34	—
24	—	—	—	—	0.33	—
25	—	—	—	—	0.32	—
26.5	—	0.54	—	—	—	—
27.5	—	—	—	0.28	—	—
32	—	0.54	—	—	—	—
32.5	—	—	—	—	—	1.065
44	—	—	—	0.29	—	—
48	—	—	0.30	—	—	—
49	—	—	0.30	—	—	—
122.5	—	—	—	—	0.11	—

* This series is not given in Fig. 2, in order to simplify the figure.

** Glycylclycin only. pH is 9.

Fig. 2. Glycylglycin + glucose.



These types of curves I shall call the "tripeptide type."

All these results together with that of dipeptides, clearly indicate, that the peptide linkage may play an important role in the interessant and regular change of order of curves.

4. ε-Aminocapronic acid + glucose (Table IV and Fig. 4).

Three different solutions at pH 7, 8 and 9 are prepared and after 4 hours the amino-N is measured.

Although the amino group and carboxyl group are far apart in ε-aminocapronic acid as in di- and tripeptide, the reaction modus belongs to the amino acid type, suggesting that the difference between the amino acid type and the di- or tripeptide type is probably due to peptide linkage.

5. Glycylglycin + galactose (Table IV and Fig. 4).

Three different solutions at pH 7, 8 and 9 are measured 1 hour and 42 minutes after their preparation. There is no significant difference between glucose and galactose.

6. Glycocoll + glucose in 50% heavy water (Table IV and Fig. 4).

Three solutions at pH 7, 8 and 9 containing 50% heavy water are prepared and after 2 hours the amino-N is measured.

The type remains the same as in the ordinary water solution, but the decrease of amino-N with the order of the value of pH is here more slight and the absolute amount of the decsease is less compared with the amount by ordinary water solution.

7. Glycylglycin+glucose in 50% heavy water (Table IV and Fig. 4).

Three solutions at pH 7, 8, and 9 containing 50% heavy

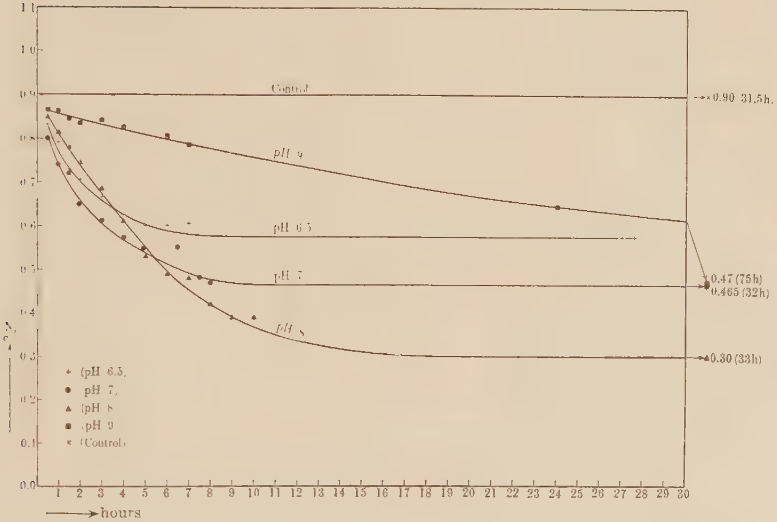
TABLE III.

1-Leucylglycylglycin+glucose (0.018 mol: 0.9 mol)

Time (hours)	N_2 cc. (760 mm, 22°C)				
	pH 6.5	pH 7	pH 8	pH 9	control*
0.2	—	—	—	—	0.90
0.5	0.83	0.80	0.845	0.865	—
1	0.79	0.74	0.81	0.86	—
1.5	0.73	0.725	0.78	0.845	—
2	0.705	0.65	0.745	0.835	—
3	0.665	0.61	0.685	0.84	—
3.5	—	—	—	—	0.90
4	0.625	0.57	0.61	0.825	—
5	0.60	0.55	0.53	—	—
6	0.60	—	0.485	0.805	—
6.5	—	0.55	—	—	—
7	0.605	—	0.48	0.785	—
7.5	—	0.48	—	—	—
8	—	0.47	0.42	—	—
9	—	—	0.39	—	—
10	—	—	0.39	—	—
24	—	—	—	0.645	—
27	0.575	—	—	—	—
27.5	0.575	—	—	—	—
31.5	—	—	—	—	0.90
32	—	0.465	—	—	—
33	—	—	0.30	—	—
75	—	—	—	0.47	—

* 1-Leucylglycylglycin only. pH is 9.

Fig. 3. l-Leucylglycylglycin + glucose.



water are prepared and after the lapse of 2 hours the amino-N is measured.

There is a slight sign of a tendency of a type of reaction towards that of the tripeptide. The fact that the type of reaction undergoes a change when the nature of the solvent is changed seems important.

8. Glycocoll + fructose (Table V and Fig. 5A).

The decrease of amino-N is here far less than that with glucose. These results agree on the whole with that of the change of optical rotatory power studied by Neuberg and his associates (l. c.).

9. Glycylglycin + fructose (Table VI and Fig. 5B).

The mode is the same as in glycocoll + fructose, the decrease of amino-N being far less than with glucose, and there is almost no sign of the dipeptide type. Though Waldschmidt-Leitz and Rauchalles (l. c.) stated that at pH 7.9 the solution containing d, l-leucylglycin and fructose shows no change in the amount of amino-N, at pH 8 in my experiment there is clearly seen a decrease as shown in Fig. 5B.

10. l-Leucylglycylglycin + fructose (Table VII and Fig. 5C).

In the case of fructose the degree of the reaction of sugar with tripeptide is very small and the disturbances in the beginning seem too slight to give any meaning.

11. Methylamine + glucose (Table VIII and Fig. 6A).

As is seen from Fig. 6A the type is exactly the same as the aminoacidic.

12. Isobutylamine + glucose (Table IX and Fig. 6B).

The type is also exactly the same as the aminoacidic. These results by amines indicate that amino acid behaves exactly in the same manner as amine.

13. Only glycocoll or glycylglycin is kept at pH 7, 8 and 9, and after the lapse of 26 hours glucose is added (Table X,

TABLE IV.

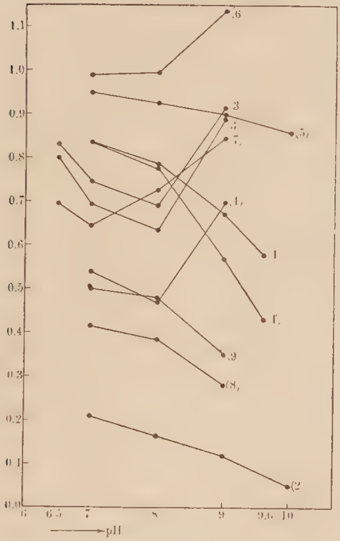
Solution (the lapse of time)	N_2 cc. (760 mm, 22° C)				
	pH 6.5	pH 7	pH 8	pH 9	pH 9.6-10
(1) Glycocoll + glucose (after 2 hours)*		0.835	0.785	0.67	0.58**
(1′) ,, (after 4 hours)*		0.835	0.72	0.57	0.43**
(2) ε-aminocapronic acid + glucose (after 4 hours)		0.21	0.165	0.12	0.05***
(3) Glycylglycin + glucose (after 1.7 hours)*	0.83	0.745	0.69	0.915	
(3′) Glycylglycin + glucose (after 2.17 hours)*	0.80	0.695	0.635	0.89	
(4) Glycylglycin + Galactose (after 1.7 hours)		0.54	0.47	0.70	
(5) Glycocoll + glucose in 50% heavy water (after 2.17 hours)		0.95	0.925	0.90	0.86***
(6) Glycylglycin + glucose in 50% heavy water (after 2.17 hours)		0.99	0.995	1.135	
(7) l-Leucylglycylglycin + glucose (after 2.17 hours)*	0.695	0.645	0.725	0.845	
(8) Methylamine + glucose (after 2 hours)		0.415	0.385	0.28	
(9) Isobutylamine + glucose (after 2 hours)		0.50	0.48	0.35	

* These values are read from the curves of Fig. 1, 2 and 3.

** These pH values are not precise.

*** These pH values are probably 10 or a little greater than 10.

Fig. 4.



(1) Glycocoll + glucose (after 2^h)
(1') ,, ,, (after 4^h)
(2) ε-Aminocapronic acid + glucose (after 4^h)
(3) Glycylglycin + glucose (after 1.7^h)
(3') ,, ,, (after 2.17^h)
(4) Glycylglycin + galactose (after 1.7^h)
(5) Glycocoll + glucose in 50% D_2O (after 2.17^h)
(6) Glycylglycin + glucose in 50% D_2O (after 2.17^h)
(7) l-Leucylglycylglycin + glucose (after 2.17^h)
(8) Methylamine + glucose (after 2^h)
(9) Isobutylamine + glucose (after 2^h)

XI and Fig. 1, 2).

In these cases all are the same as in the case in which glycocoll or glycylglycin and glucose are dissolved at the same time. This fact, together with the fact that the amount of amino-N of glycocoll or glycylglycin does not suffer any change when kept at pH 9 during 32 hours, shows clearly that there may be no change on the side of glycocoll or glycylglycin itself during the whole period of experiment, although there is seen a slight sign of displacement towards an increase of change in the case of pH 7 and a decrease in the case of pH 9, when compared with the case of direct mixing.

14. Only glucose is kept at pH 7, 8 and 9 and after the lapse of 26 or 20 hours glycocoll or glycylglycin is added respectively. (Table XII, XIII).

In these cases all remains exactly the same as in the case in which both components are dissolved simultaneously.

This fact denotes that any change on the side of sugar, if occuring, did not affect in the least the mode of the curve, at least under conditions adopted here. That some changes occur

TABLE V.

Glycocoll + fructose (0.018 mol : 0.9 mol)

Time (hours)	N_2 cc. (760 mm, 22°C)			
	pH 7	pH 8	pH 9	control*
0.2	—	—	—	0.86
0.5	0.855	0.85	0.83	—
1	0.86	0.855	0.83	—
1.5	—	0.85	—	—
3	—	0.85	0.83	—
4	—	—	—	0.855
4.5	0.855	—	—	—
6	—	0.85	—	—
7	—	—	0.82	—
9	0.855	—	—	—
30	—	0.85	0.795	—
31	0.855	—	—	—
32	—	—	—	0.86

* Glycocoll only. pH is 9.

TABLE VI.

Glycylglycin + fructose (0.018 mol : 0.9 mol)

Time (hours)	N_2 cc. (760 mm, 22°C)			
	pH 7	pH 8	pH 9	control*
0.2	—	—	—	1.07
0.5	1.07	1.025	1.00	—
1	1.025	—	1.00	—
1.5	1.035	1.04	—	—
2	1.02	1.02	1.00	—
3	1.04	1.03	1.01	—
4	—	—	—	1.07
5	1.035	—	—	—
6.5	—	1.01	—	—
23	—	—	0.98	—
24.5	—	1.02	—	—
32.5	1.04	—	—	1.065

* Glycylglycin only. pH is 9.

TABLE VII.
1-Leucylglycylglycin + fructose (0.018 mol : 0.9 mol)

Time (hours)	N_2 cc. (710 mm, 22°C)			
	pH 7	pH 8	pH 9	control*
0.2	—	—	—	0.90
0.5	0.885	0.895	0.89	—
1	0.875	0.87	0.88	—
2	0.885	—	0.865	—
3	—	0.87	0.865	—
3.5	—	—	—	0.90
8	—	—	0.85	—
9	—	—	0.855	—
16.5	—	0.87	—	—
19	0.88	—	—	—
23	—	—	0.83	—
31.5	—	—	—	0.90

* 1-Leucylglycylglycin only. pH is 9.

Fig. 5. (A) Glycocoll + fructose.
(B) Glycylglycin + fructose.
(C) 1-Leucylglycylglycin + fructose.

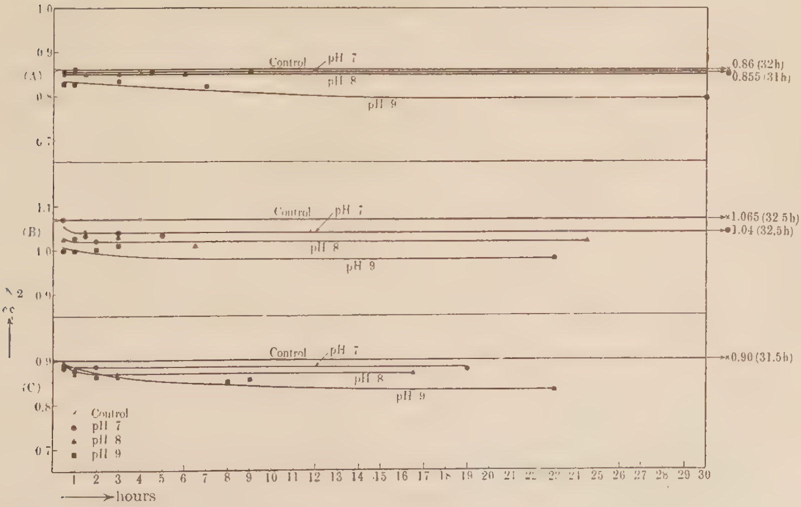


TABLE VIII.

Metylamine + glucose (0.009 mol: 0.9 mol)

Time (hours)	N_2 cc. (760 mm, 22°C)				
	pH 7	pH 8	pH 9	control*	pH 9→8**
0	—	—	—	—	0.265
0.2	—	—	—	—	0.29
0.5	0.43	0.58	0.35	0.46	0.33
1	0.40	0.38	0.32	—	0.33
1.5	0.40	—	—	0.43	0.39
2	—	—	0.25	—	0.39
3	—	—	0.275	—	—
5	—	0.39	—	—	—
15	—	—	—	0.44	—
16	0.415	—	—	—	0.395
21	—	—	0.265	—	—
23	—	0.385	—	—	—
30	—	—	0.265	—	—

* Methylamine only. pH is 9.

** This series is for the experiment to see reversibility.

TABLE IX.

Isobutylamine + glucose (0.009 mol : 0.9 mol)

Time (hours)	N_2 cc. (760 mm, 22°C)			
	pH 7	pH 8	pH 9	control*
0.5	0.50	0.50	0.435	0.51
1	0.50	—	0.40	0.49
2	—	0.48	0.345	—
3	0.50	0.48	0.335	—
5	—	0.48	—	—
6	—	—	0.335	—
20.5	—	—	—	0.525
21	0.495	—	—	0.525
21.5	—	0.49	—	—
50	—	—	0.305	—

* Isobutylamine only. pH is 9.

Fig. 6. (A) Methylamine + glucose.
(B) Isobutylamine + glucose.

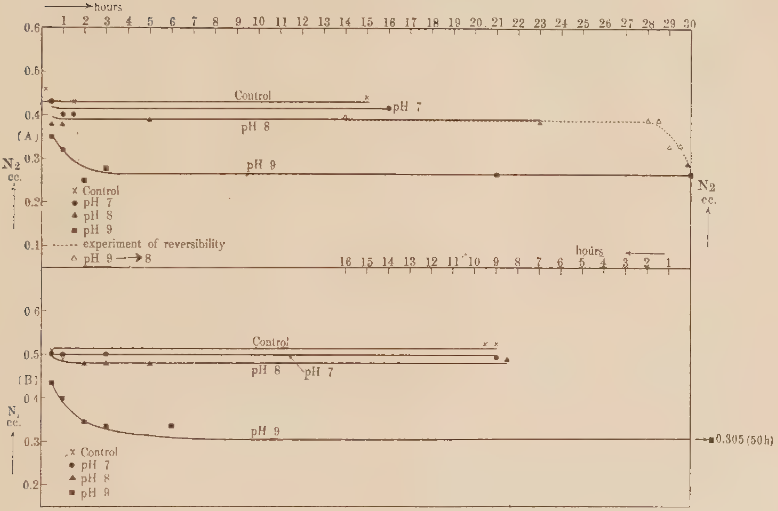


TABLE X.

Glycocoll + glucose (0.018 mol; 0.9 mal)*

Time (hours)	N_2 cc. (760 mm, 22°C)				
	pH 7	pH 8	pH 9	pH 9·1**	control***
0.2	—	—	—	—	0.86
0.5	0.855	0.82	0.805	0.80	—
1	0.825	0.795	0.76	0.73	—
2	0.835	0.77	0.72	0.645	—
4	—	—	—	—	0.855
24.5	—	0.78	—	—	—
27	—	—	—	0.43	—
29.5	0.835	—	—	—	—
32	—	—	—	—	0.86
50	—	—	0.48	—	—
51	—	—	0.48	—	—

* This series is given in Fig 1. by dotted lines.

** For the sake of simplicity this is not plotted in Fig. 1.

*** Glycocoll only. pH is 9.

TABLE XI.

Glycylglycin + glucose (0.018 mol : 0.9 mol)*

Time (hours)	N_2 cc. (760 mm, 22°C)			
	pH 7	pH 8	pH 9	control**
0.2	—	—	—	1.07
0.5	0.88	0.91	0.995	—
1	0.81	0.815	0.97	—
2	0.69	0.66	0.91	—
4	—	—	—	1.07
18	—	0.30	—	—
21.5	—	—	0.38	—
23.5	0.54	—	—	—
32.5	—	—	—	1.065

* This series is given in Fig. 2. by dotted lines.

** Glycylglycin only. pH is 9.

TABLE XII.

Glucose + glycocoll (0.9 mol : 0.018 mol)*

Time (hours)	N_2 cc. (760 mm, 22°C)			
	pH 7	pH 8	pH 9	control**
0.2	—	—	—	0.86
0.5	0.85	0.85	0.795	—
1	0.84	0.845	0.745	—
2	0.835	—	0.655	—
2.5	—	0.78	—	—
4	—	—	—	0.855
17.5	—	0.785	—	—
20	—	—	0.48	—
22.5	0.835	—	—	—
32	—	—	—	0.86

* In this experiment the decrease of amino-N is almost exactly the same as in the case where both components were dissolved at the same time; therefore, for the sake of simplicity, these are not plotted in Fig. 1.

** Glycocoll only pH is 9.

TABLE XIII.

Glucose + glycylglycin (0.9 mol : 0.018 mol)

Time (hours)	N_2 cc. (760 mm, 22°C)			
	pH 7	pH 8	pH 9	control**
0.2	—	—	—	1.07
0.5	0.895	0.93	0.99	—
1	0.83	0.845	0.93	—
2	0.69	0.81	0.90	—
4	—	—	—	1.07
25.5	—	—	0.33	—
26	—	0.30	—	—
32.5	0.535	—	—	1.065

* For simplicity's sake this series is not given in Fig. 2.

** Glycylglycin only. pH is 9.

on sugar which do not influence the results of these experiments, may be supposed from the fact that pH value of all kinds of solutions adjusted to pH 9 and 8 shows a very slight, yes, generally a hardly noticeable tendency to decrease in the course of time.

15. Reversibility of the reaction (Table VIII, and Fig. 6A).

The reversibility of the reactions is examined in the case of methylamine + glucose, for example. After the lapse of 30 hours the solution of pH 9 is adjusted to pH 8 and succeeding determinations were made on the amount of the amino-N. As is clear from the table and figure, the amount of amino-N begins to increase and, after about 2 or 3 hours the equilibrium is attained exactly the same as in the case where the solution is kept at pH 8 from the beginning.

This result may indicate that in all cases where the equilibrium is attained at least, the reaction taking place is reversible.

IV. DISCUSSION AND CONCLUSION.

Taking all facts described above into consideration, the types of reaction of amino acids, dipeptide or tripeptide with glucose are characteristic for themselves. In the case of amino acids, the amount of the decrease of amino-N runs parallel with the value of pH of the solution. It seems reasonable to suppose in this case that amino acid combines by its amino group with the aldehyde group of glucose, forming a methylene-like compound, simply facilitated by the higher value of pH by increasing the concentration of the free amino group without electrical charge, though the nature of the combination itself is not yet clear even in the case of amino acid and formaldehyde (Levy: 1932, 1935, 1937; Tomiyama: 1935; Balson and Lawson: 1936). It is noticeable that the reaction is very slow, and at pH 9 about 10 hours were required before the equilibrium is attained, and that the reaction is probably reversible.

In the case of glycylglycin, the amount of combination at pH 9 is less than that at pH 7 and 8 until the lapse of about 12 hours. After about 28 hours the relation between the amount of combination and pH becomes the same as that of amino acid glucose mixture. In the case of l-leucylglycylglycin this reversion to the amino acidic type is altogether not attained even after about 75 hours. These changes of relation between the amount of combination and pH may be due to the peptide linkage because of the fact that this change runs parallel with the number of peptide linkage.

These differences between amino acid and peptide can not be understood either from the difference of their isoelectric points, or from the difference in the ratio of Zwitterion and absolutely neutral molecule without any electrical charge between peptide and amino acid as calculated by the calculations of Edsall and Blanchard (1933), according to whom the ratio is 263000 by glycocoll and 40740 by glycylglycin.

From these considerations we are forced to examine the mechanism of reaction of amino acid or peptide with sugar and also to give some consideration to the structure of amino acid and peptide. Concerning these points the result obtained in 50% heavy water solution is very suggestive. In the immediately succeeding report I shall undertake this problem.

Just a few words more concerning the case of ketose. It is different from the case of aldose, that with ketose the amount of the combination is very little, and the type in dipeptide remains nearly equal as in amino acid, while in tripeptide a trace of the sign of the peptide type appears. The profound differences existing between aldose and ketose may contribute to elucidate the nature of the reaction of amino compound with sugar.

V. SUMMARY.

1. By means of van Slyke's amino-N measuring method the combination between amino acid or peptide with glucose at pH 7, 8 and 9 are investigated during a period of 1 or 2 days.

2. In the case of amino acid the amount of combination runs parallel with pH.

In the case of glycylglycin, on the contrary, the amount of combination at pH 9 is less than that at pH 7 and that at pH 8, until the lapse of about 12 hours and about 28 hours respectively.

In the case of l-leucylglycylglycin, the amount of combination at pH 9 is less than that at pH 7 even after the lapse of 75 hours, and, moreover, during the first 5 or 6 hours the amount at pH 8 is also less than that at pH 7.

3. When fructose is used in place of glucose, the amount of combination is far less than with the case of glucose.

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STUDIEN ÜBER DIE BEDEUTUNG DES EISEN-PORPHYRINS IM ZELLSTOFFWECHSEL. I.

Eisen-Porphyringehalt und Atmungsfähigkeit der verschieden genährten Hefen.

Von

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In den letzten Jahren ist von verschiedenen Seiten immermehr aufgeklärt worden, dass der Eisen-Porphyrinkörper in Mechanismus der Zellatmung eine ungemein grosse Rolle spielt.

In Muskeln und anderen Geweben von Tieren der verschiedenen Ordnungen des Tierreiches fand Mac Munn (1886) einen Farbstoff mit hämochromogenähnlichem Spektrum, den er für einen Atmungsfarbstoff hielt und Myohämatin oder Histohämatin nannte. In neuerer Zeit stellte Keilin (1925) grosse Verbreitung dieser Stoffe nicht nur im Tier-, sondern auch im Pflanzenreich fest und ersetzte die Bezeichnung Mac Munn's durch den allgemeineren Namen "Cytochrom". In weiteren Arbeiten (1929/30) wurde dann die Eisen-Porphyrinnatur dieses Farbstoffs bestätigt und seine Funktionen zu ergründen versucht. Keilin ist bekanntlich zu der Ansicht gekommen, dass Cytochrom zwischen den Dehydrasen, die den Wasserstoff der Nährstoffe aktivieren, und den Oxydasen, die den molekularen Sauerstoff aktivieren, als "Carrier" fungiert. Seither sind in dieser Richtung durch mehrere Forscher ausführlichere Bearbeitungen fortgesetzt worden.

Ausser Keilin vertritt besonders Warburg die Auffassung, dass das Porphyrin-Eisen als ein sauerstoffaktivierendes Zentrum bei den Atmungsvorgängen in der Zelle wirkt. Während er anfangs dem unorganischen Eisen die Rolle eines die Oxydation vermittelnden Katalysators erteilte, wurde er gestützt auf seine elegante, photochemische Untersuchung betreffs der Lichtempfindlichkeit der CO-Verbindung des Atmungsfermentes zu der Annahme geführt, dass dieses ein Eisen-Porphyrin sei, das in seinen spektroskopischen Eigenschaften dem Phäohämin *b* und dem Spirographishämin nahe liegt. Laut ihm soll der Hauptteil oder mindestens der cyanempfindliche Teil der Sauerstoffübertragung in der Zelle durch diese Eisen-Porphyrinkatalyse vermittelt werden (Warburg, Negelein, 1929; vgl. auch Reid, 1932). Später fanden Warburg, Negelein und Haas, dass Bac. Pasteurianum in konzentrierter Suspension ein schwaches Absorptionsband auf 589 $m\mu$ aufweist, und wegen der Verschieblichkeit des Bandes bei Sättigung der Suspension mit Kohlenoxyd meinten sie, dass die Bänder von "Atmungsferment" herrühre (1933). Dagegen ist aber heute die Ansicht sichergestellt, dass dieser Farbstoff nichts anders als eine neue Komponente des Cytochroms zu betrachten ist (Keilin, 1933).

Hier sei noch erwähnt, dass sowohl die Peroxydase (Kuhn, 1931) als auch die Katalase (Zeile, Hellström, 1930; Zeile, 1931) sehr wahrscheinlich ein Eisen-Porphyrin und zwar Protohämin als einen unentbehrlichen Bestandteil besitzen. Wenn auch die Wirkungsweise der beiden Fermente im Organismus heute noch nicht völlig aufgeklärt ist, so ist es durchaus zweifellos, dass diese beim Zellstoffwechsel irgendeine wesentliche Rolle spielen.

Aber um die Frage nach der Bedeutung des Eisen-Porphyrins in Zelle noch ausführlicher aufzuklären, scheint es mir nötig zu sein, die Verhältnisse genau auf quantitative Weise zu erforschen. Zuerst kann man besser die Mengenveränderung des Eisen-Porphyrins unter verschiedenen Bedingungen bei dem betreffenden Lebewesen studieren und sie mit seinen anderen biologischen Eigenschaften in Beziehung setzen. Mit dieser Absicht habe ich die folgenden Experimente ausgeführt, in denen ich die Bäckerhefe als das geeignete Versuchsmaterial verwendete.

I. BESTIMMUNGSMETHODE DES EISEN-PORPHYRINGEHALTES.

Zu meinem Zweck kamen die Hämochromogene im Pyridinextrakt in Betracht. Nachdem schon vor der Entdeckung des Cytochroms Fischer und Hilger (1924) als erste Hämatin und Hämochromogen aus Hefe spektroskopisch beobachtet hatten, bereiteten Anson und Mirsky (1925) Pyridinextrakte aus Hefe und bestätigten, dass der intensivste Absorptionsstreifen seiner Lage nach vollständig mit dem α-Band des Pyridinhämochromogens von Protohämin übereinstimmt. Ein endgültiger Beweis für seine Identität mit Protohämin ist später insofern erzielt worden, als Fischer und Schwerdtel (1928) es aus Pyridinextrakt in krystallisiertem Zustand gewannen.

Für die quantitative Bestimmung des Protohämins im Pyridinextrakt aus Hefe sind zwei spektroskopische Methoden angegeben worden (Treibs, 1927; Euler, Fink, Hellström, 1927). Aber mit diesen beiden spektrophotometrischen Messmethoden können gewisse Ungenauigkeiten verursacht werden, da auch die gelben bzw. braunen undefinierbaren Farbstoffe in den Pyridinextrakt mitgehen und ebenfalls Licht absorbieren; überdies ist es wünschenswert, das mit Pyridin extrahierbare Eisen-Porphyrin insgesamt zu messen.

Nach zahlreichen Versuchen, den reduktiven Abbau des Eisen-Porphyrins bis zum Pyrrole zu seiner quantitativen Bestimmung zu benutzen, erwies sich das folgende Verfahren, wenn auch nicht so vollkommen ausgearbeitet, zweckmässig:

Eine abgewogene Menge der gut trocken abgesaugten Hefe wird auf Glasfilter gebracht, mit Pyridin, welches nach Treibs (1927) durch Behandeln mit Chromsäure und Kali gereinigt ist, und dem eine kleine Menge Natriumsulfit und Cyankalium zugesetzt ist übergossen, und durch Zerdrücken bis zur völligen Suspension zerteilt. Da die Hefe von Pyridin äusserst energisch unter Wärmeentwicklung plasmolysiert wird, und da dabei Eisen-Porphyrin in Pyridin sehr leicht übergeht, wird die Suspension nach 15 bis 20 Minuten langem Verrühren durch Glasfilter abgesaugt. Der Filtrierrückstand wird noch zweimal mit

Pyridin ausgezogen. Der Extrakt wird in einem kleinen Destillationskolben aufgenommen und das Pyridin im Vakuum abdestilliert.

Alle eben erwähnten Manipulationen müssen wegen der grossen Empfindlichkeit des Hämochromogens gegen Luftsauerstoff stets im Stickstoffmedium ausgeführt werden.

Der sirupöse Rückstand wird mit 10 ccm neutralem Essigäther übergossen und durch Asbestfilter filtriert, das Filter wird nochmals mit 10 ccm Essigäther gewaschen. Das vereinigte Filtrat wird in einen Scheidetrichter von 50 ccm Inhalt getan und mit 10 ccm Sulfit-Cyanid-Lösung (0,8 g wasserfreies Natriumsulfit und 0,2 g Kaliumcyanid in 100 ccm Wasser) zweimal extrahiert. Die Farbstoffe lassen sich durch die erstmalige Extraktion fast vollständig zur Sulfit-Cyanid-Lösung überführen. Jede Fraktion des Extrakts wird dann durch ein kleines Filtrierpapier in ein Zentrifugenglas von 15 ccm Inhalt filtriert, zu der nachher 1 ccm 0,4%-iges Ammoniakwasser und 1 ccm 5%-ige $ZnSO_4 \cdot 7H_2O$-Lösung unter ständigem Rühren zugefügt werden. Nach Stehen über eine Nacht wird der Niederschlag, der die Farbstoffe vollkommen mitgerissen hat, scharf zentrifugiert.

Zu dem Niederschlag werden nun 2 ccm Salzsäure-Eisessig (1 Vol. 38% Salzsäure + 2 Vol. Eisessig) gefügt, und der Niederschlag wird unter Umrühren mit einem feinen Glasstab gelöst. Die Lösung wird dann mit Hilfe von 4 ccm Salzsäure-Eisessig in einen kleinen Rundkolben übergeführt. 0,5 ccm 0,2%-ige $CuCl_2 \cdot 2H_2O$-Lösung in Eisessig wird hinzugefügt und darauf werden vier 1 cm lange von einem Zinkstab (Kahlbaum: Chem. rein in Stangen 3 mm ϕ "zur Analyse") geschnittene Stücke hineingetragen. Nach einstündigem Stehen unter Rückflusskühler in siedendem Wasserbad wird die Flüssigkeit abgegossen, das Zink mit möglichst wenigem Wasser gewaschen und die Waschflüssigkeit mit dem ersten Abguss vereinigt.

Zu der Flüssigkeit setzt man 0,3 ccm salzsaure p-Dimethylaminobenzaldehydlösung (p-Dimethylaminobenzaldehyd 0,2 g, 38% Salzsäure 4 ccm mit destilliertem Wasser auf 100 ccm) zu, worauf eine rosa-rote Farbe der Pyrrolreaktion eintritt. Da die

Farbe nach zwei bis drei Stunden völlig entwickelt verdünnt man alsdann mit Wasser auf 40 ccm, schüttelt den Farbstoff mit 10 ccm reinem Amylalkohol aus, lässt die Mischung einige Zeit ruhig stehen und vergleicht die klare Amylalkoholschicht mit einer wässerigen Fuchsin-Phenosafraninlösung (0,01%-ige Lösung von Fuchsin-Grübler und 0,01%-ige Lösung von Phenosafranin-Grübler werden zu gleichen Volumenteil vermischt und mit Wasser bis auf passende Konzentration in bekanntem Verhältnis verdünnt).

Versuche mit reinem Hamin.

Um von der gemessenen Farbenintensität der Pyrrolreaktion auf den ursprünglichen Gehalt an Eisen-Porphyrin schliessen zu können, verglich ich die Farbenintensität mit der zur Bestimmung kommenden Menge des reinen Cl-Hämin, welches aus Rinderblut nach Schalfejew, Nencki und Zaleski dargestellt und nach dem Pyridin-Chloroform-Verfahren von Küster (1922) umkristallisiert wurde.

1 ccm Häminlösung mit bekannter Konzentration wurde wie oben der reduktiven Spaltung mit Salzsäure-Eisessig und Zink unterworfen. Die weitere Behandlung war ganz ebenso wie bei der obenerwähnten eigentlichen Bestimmungsmethode. Die Resultate sind in Tabelle I angegeben. Die Kurve, deren Abszissen die Häminmengen in Milligramm und deren Ordinaten die Ver-

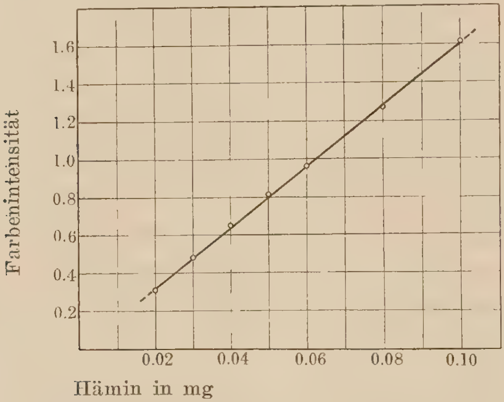
TABELLE I.

Proportionalität zwischen Häminmenge und Farbenintensität

Hämin in mg	Farbenintensität			
	(1)	(2)	(3)	Durchschnitt
0,1	1,71	1,64	1,49	1,61
0,08	1,27	1,31	1,24	1,27
0,06	0,96	0,93	0,98	0,96
0,05	0,80	0,81	0,81	0,81
0,04	0,64	0,67	0,65	0,65
0,03	0,48	0,47	0,48	0,48
0,02	0,30	0,33	0,31	0,31

Fig. 1.

Proportionalität zwischen Häminmenge und Farbenintensität.



hältnisse der Farbenintensitäten sind, ist in Fig. 1. dargestellt.

Die Kurve zeigt nur geringe Abweichungen von der Geraden, und es erweist sich, dass zwischen kolorimetrischer Ablesung und Häminmenge so genügende Proportionalität besteht, dass die Menge des Eisen-Porphyrins in Pyridinextrakt aus Hefe mittels dieser Messmethode als Protohämin ohne besondere Schwierigkeiten berechnet werden kann.

Prüfung der Methode durch Additionsversuch.

Um die Anwendbarkeit meiner Methode zur Bestimmung des Eisen-Porphyrins im biologischen Material zu prüfen, habe ich in erster Linie Additionsversuche ausgeführt. Zuerst wurde nach meiner Methode der Eisen-Porphyringehalt in Pyridinextrakt aus der abgewogenen Menge Hefe bestimmt. Darauf wurde der Pyridinextrakt aus derselben Menge der Hefe mit einer bestimmten Menge Hämin versetzt und wie oben wieder das Eisen-Porphyrin bestimmt.

Die Bestimmungen führten zu den in der Tabelle II zusammengefassten Ergebnissen. Die Zahl der Eisen-Porphyrinmenge ist als Protohämin berechnet.

Aus der Tabelle geht hervor, dass die Gesamt-Eisen-Porphyrinmengen mit einem unter 2% liegenden Fehler wiedergefunden

TABELLE II.

Additionsversuche.

Versuch Nr.	Eisen Porphyrin in Pyridinextrakt in mg	Häminzusatz in mg	Gesamt-Eisen-Porphyrin		Fehler %
			berechnet in mg	gefunden in mg	
1	0,0293	0,0212	0,0505	0,0494	−2,2
2	0,0291	0,0212	0,0503	0,0503	0
3	0,0292	0,0212	0,0504	0,0502	−0,4
4	0,0442	0,0212	0,0654	0,0645	−1,4
5	0,0422	0,0212	0,0634	0,0640	+1,0

wurden, also mit durchaus zufriedenstellender Genauigkeit.

Anwendung der Methode auf Presshefe.

2,5 g käufliche Bäckerhefe wurden auf Glasfilter gebracht, nach der Zugabe von 0,1 g wasserfreiem Natriumsulfit und 0,005 g Cyankalium mit 20 ccm gereinigtem Pyridin in sauerstofffreier Atmosphäre gut verrührt; die Suspension wurde nach 15 bis 20 minutenlangem Verrühren abgesaugt und der Filtrierrückstand noch zweimal mit je 5 ccm Pyridin ausgezogen.

Der Extrakt färbt sich orange-rot und zeigt spektroskopisch den deutlichen Hauptabsorptionsstreifen des Hämochromogens 557 mμ, und daneben einen schwachen Absorptionsstreifen auf ungefähr 586 mμ, den Anson und Mirsky (1925) für den von Cytochrom a herrührenden Streifen gehalten haben. Im Gegensatz zu Fischers Angabe, dass die Hämochromogene in dem Pyridinextrakt aus Hefe äusserst hinfällig sind, waren die Absorptionsspektren unter den beschriebenen Bedingungen durchaus beständig und auch nach längerer Zeit liess sich keine merkliche Abschwächung der Spektren wahrnehmen.

Das Pyridin wurde dann in Vakuum abdestilliert und durch weitere Behandlungen wurde die Eisen-Porphyrinmenge bestimmt. Es wurden vier Bestimmungen für dieselbe Hefeprobe durchgeführt; die erhaltenen Werte für 2,5 g Hefe sind

0,0433 mg
0,0443

0,0441
0,0424
Durchschnitt: 0,0435

Also die Übereinstimmung der Werte ist durchaus befriedigend.

In der Tabelle III sind Trockensubstanzmenge, Eisen-Porphyringehalt und Gesamt-Eisengehalt bei unterschiedlichen Materialien der käuflichen Bäckerhefe zusammengestellt. Der Gehalt an Eisen wurde nach der Methode von Kennedy (1927) bestimmt. Aus der Tabelle III ist ersichtlich, dass in Hefezellen nur ein kleiner Bruchteil des Gesamt-Eisens als Porphyrin-Eisen enthalten ist.

Tabelle III.

Mit Pyridin extrahierbares Eisen-Porphyrin und Gesamt-Eisen der Käuflichen Bäckerhefe.

Hefe Nr.	Datum des Versuches	Trockensubstanz %	Eisen-Porphyrin		Gesamt-Eisen		Porphyrin-Eisen: Gesamt-Eisen
			für 1g frische Hefe in γ	für 1g Trockensubstanz in γ	für 1g frische Hefe in γ	für 1g Trockensubstanz in γ	
1	20 IV	26.6	5.7	21.3	107	417	1:230
2	2 V	24.2	4.5	18.7	98	405	1:253
3	24 V	25.9	7.3	28.0	136	527	1:220
4	31 V	25.8	6.3	24.5			
5	7 VI	28.2	7.7	27.2	132	468	1:202
6	16 IX	26.4	5.2	19.7	114	421	1:249
7	25 IX	31.1	5.8	18.5	102	328	1:207

II. Einfluss von Eisen- und Kupferzusatz zur Nährlösung auf den Eisen-Porphyringehalt der Hefe.

Im Jahre 1931 hat Elvehjem den Einfluss des Eisens und des Kupfers auf die Hefe mitgeteilt. In der möglichst von Eisen und Kupfer befreiten synthetischen Nährlösung wächst die Hefe schlecht und die geerntete Hefe zeigt nur schwache Absorptionsstreifen des Cytochroms. Dagegen in der mit einer kleinen Menge Eisens ergänzten Nährlösung wird das Wachstum in hohem Masse befördert und zugleich nimmt der Gehalt an

Cytochrom stark zu. Gleichzeitige Zugabe von Eisen und Kupfer begünstigt das Wachstum weiter und stimuliert die Produktion des Cytochroms mit besonderem hohen Gehalt an a-Komponent.

Die folgenden Untersuchungen beschäftigen sich mit dem Studium über den Einfluss von Eisen und Kupfer auf die Synthese des Eisen-Porphyrins in der unter bestimmten Bedingungen gezüchteten Bäckerhefe.

1. *Das Verfahren zur Befreiung der Nährlösung vom Eisen.*

Was mit der Reinigungsmethode zu erreichen war, stellte ich mit der Rhodanidprobe auf Eisen fest, und zwar verwendete ich zur Prüfung eine 20% Natriumrhodanidlösung, die wegen ihres eigenen, relativ hohen Eisengehaltes vor dem Gebrauch nach Zusatz von einigen Tropfen reiner Salzsäure wiederholt durch reinen Amylalkohol ausgeschüttelt wurde, bis die Amylalkoholschicht sich nicht mehr rot färbte. Mit 5 ccm einer so gereinigten Rhodanidlösung versetzte ich 20 ccm der zu prüfenden Flüssigkeit; nach Zusatz von Salzsäure und Wasserstoffperoxyd wurde sie mit 2 ccm Amylalkohol ausgeschüttelt. Durch diese Methode konnte ich 0,2 γ Eisen deutlich nachweisen.

Um eine möglichst vollkommen von Eisen befreite Nährlösung zu bekommen, wurden zunächst die einzelnen Nährsalze und der Zucker gereinigt:

1. *Ammoniumchlorid.* 500 g Ammoniumchlorid wurden in 1,5 *l* Wasser gelöst, mit Salzsäure etwas angesäuert und in diese Lösung wurde, nach Zugabe einer kleinen Menge von Tierkohle, Schwefelwasserstoff eingeleitet. Nach einigen Stunden wurde die Flüssigkeit durch ein Faltenfilter filtriert, darauf mittels Ammoniakwassers schwach alkalisch gemacht und unter erneuter Zugabe von Tierkohle wieder filtriert. Das Filtrat wurde dann nach dem Ansäuern mit Salzsäure aufgekocht, um den Schwefelwasserstoff auszutreiben, und nach nochmaligem Filtrieren zur Kristallisation eingedampft. Die so gewonnenen Kristalle waren fast rein aber noch nicht vollkommen von Eisen befreit. Zur Beseitigung der letzten Spur des Eisens war die

von Bertrand angegebene Methode zur Entfernung der Manganverunreinigung der Salze zweckmässig. Das Salz wurde in der dreifachen Menge reinen Wassers gelöst, nach Zufügen von mehreren Tropfen Wasserstoffperoxyd auf etwa 50°C erwärmt. Die Flüssigkeit wurde mit Ammoniakwasser alkalisiert und dann mit je m/100 Magnesiumchlorid und Ammoniumphosphat versetzt. Vom Niederschlag, der den Rest des Eisens mitgerissen hatte, wurde die Lösung durch ein aschenfreies Filterpapier abfiltriert und auf dem Wasserbade bis zum Kristallisieren verdunstet.

2. *Kaliumchlorid.* 500 g des Salzes wurden mit 1,5 *l* kaltem Wasser verrieben, durch ein Faltenfilter filtriert und nach Zusatz von einigen Tropfen Wasserstoffperoxyd gelinde erhitzt. Hierauf wurde eine aus 5 g gebranntem Kalk bereitete Kalkmilch zugesetzt. Nachdem der Niederschlag sich zu Boden gesetzt hatte, wurde die Lösung filtriert und die klare Flüssigkeit mit einer Lösung von 15 g reinem, wasserfreiem Kaliumkarbonat gefällt. Das Filtrat wurde in der Siedehitze mit Salzsäure neutralisiert und möglichst schnell auf etwa 0,5 *l* eingedampft. Das abgeschiedene Kaliumchlorid wurde abgesaugt und durch Erhitzen getrocknet.

3. *Magnesiumsulfat.* Eine konzentrierte Lösung von Magnesiumsulfat wurde auf 40 bis 50°C erwärmt und eine geringe Menge des Wasserstoffperoxyds zugefügt. Zu der erkalteten Flüssigkeit wurde in kleinen Mengen soviel Magnesiumoxyd, welches zuvor mit Wasser zu einem homogenen Brei angerieben war, zugesetzt bis dasselbe nach dem Umschütteln nicht mehr gelöst wurde. Nachdem die Mischung unter zeitweiligem Umschütteln 12 Studen lang gestanden hatte, wurde zunächst eine Probe der Lösung abfiltriert und dieselbe mit der Rhodanidprobe auf Eisen geprüft. Trat keine Rotfärbung ein, so wurde die Flüssigkeit filtriert, und nach Zusatz reiner, eisenfrei destillierter Schwefelsäure zur Kristallisation eingedampft. Trat indessen noch eine Eisenreaktion ein, so wurde von neuem etwas Magnesiumoxydbrei zugefügt und damit die Lösung abermals 12 Stunden digeriert.

4. *Natriumphosphat.* 1 kg kristallinisches Natriumphosphat wurde mit 500 ccm Wasser übergossen und in der Hitze gelöst. Zu der noch warmen Flüssigkeit wurde, nach Zufügen einer geringen Menge Wasserstoffperoxyds, Kalkmilch in kleinen Portionen mehrmals zugesetzt und jedesmal unter stetigem Erwärmen die Mischung digeriert. Wenn die abfiltrierte Probe der Mischung keine Eisenreaktion mehr zeigte, so wurde die ganze Lösung warm filtriert und zur Auskristallisation abgekühlt.

5. *Calciumchlorid.* Das Salz kam als eine wässerige Lösung zum Gebrauch. Eine 15%-ige Lösung von Calciumchlorid wurde mit einer kleiner Menge gebrannten Kalks einige Zeit aufgekocht und darauf einige Tropfen Wasserstoffperoxyd zugegeben. Nachdem die Flüssigkeit 12 Stunden lang gestanden hatte, wurde sie in einen Kolben filtriert. Zu der klaren Flüssigkeit wurde dann tropfenweise soviel 1% Phosphorsäure zugesetzt, bis eine deutliche Trübung (Fällung des Calciumphosphats) erschien. Sodann wurde die Flüssigkeit noch einige Minuten erhitzt, damit der Phosphatniederschlag sich grobkörnig absetzte, und nach dem Erkalten durch ein aschenfreies Filter abfiltriert. Das Filtrat, welches keine Rhodaneisenreaktion mehr zeigte, wurde mit reiner Salzsäure neutralisiert, und sein Gehalt an Calciumchlorid mittels der Silbernitratlösung titriert.

6. *Rohrzucker.* Zu einer konzentrierten Lösung Rohrzuckers (spez. Gew.: 1, 32) wurde zweifaches Volumen von redestilliertem Methanol, welches zuvor mit eisenfreier Salzsäure bis zur HCl-Konzentration von rund 0,1% angesäuert war, unter beständigem Umrühren zugefügt. Zur Vollendung der bald auftretenden Ausscheidung der Kristalle wurde es einige Tage bei kühler Aussentemperatur stehen gelassen. Der auskristallisierte Zucker wurde abgesaugt und zunächst mit salzsaurem, dann mit reinem Methanol ausgewaschen.

Der so gereinigte Zucker war aber noch nicht genügend eisenfrei (etwa 5γ Eisen in 25 g Zucker), und dreimalige Wiederholung der Umkristallisation erzielte nicht die vollkommene Befreiung von Eisen. Deshalb habe ich die Wiederholung der Umkristallisation aufgegeben und um eine genügend reine Nährlösung zu bereiten, verfuhr ich folgendermassen:

Herstellung der reinen Nährlösung: 25 g obenerwähnterweise umkristallisierter Rohrzucker und 6,5 g kristallinisches Natriumphosphat (=2,5 g Na_2HPO_4) wurden in 1 *l* redestilliertem Wasser gelöst und nach Zusatz kleiner Menge Wasserstoffperoxyd wurde tropfenweise 0,5 ccm 5%-ige Calciumchloridlösung unter Umschwenken zugefügt. Die Lösung wurde über Nacht stehen gelassen und dann durch ein aschenfreies Filter abfiltriert, wobei der Niederschlag von Calciumphosphat fast vollständig die Eisenverunreinigung aus der Lösung mitgerissen hatte. In diese Lösung wurde 2,5 g KCl, 2,5 g NH_4Cl, 2,5 g $MgSO_4$ und 0,5 g $CaCl_2$ eingebracht und alsdann wurde die Acidität der Lösung mit der bis zu vollkommener Eisenfreiheit destillierten Salzsäure auf pH=5,8 gebracht. 1 *l* dieser Nährlösung wurde in den Kulturkolben von 1,5 *l* Inhalt eingegossen und zur Sterilisierung einige Zeit aufgekocht. Alle Glasapparate waren vor dem Gebrauch mit Chromschwefelsäure und Aqua dest. weitgehend gereinigt worden. Wenngleich auf diese Weise noch längst nicht die letzte Spur Eisen aus der Lösung beseitigt wurde (rund 2γ Eisen in 1 *l* Lösung), genügte jedoch dieses Verfahren vollkommen, um damit die Wirkung des Eisens auf Hefezellen zu untersuchen.

2. *Einfluss des Eisens auf die Entwicklung und den Eisen-Porphyringehalt der Hefe.*

Im folgenden Versuche wurde zunächst je 0,5 g Hefe, entsprechend dem Trockensubstanzgewicht von 0,13–0,14 g, in die Kulturkolben mit 1 *l* obiger reinen Nährlösung geimpft und unter stetiger Durchlüftung 43 bis 44 Stunden lang in einem Brutschrank von 25°C gestellt. Am Schluss der Bebrütungszeit wurde die geerntete Hefe von der Nährlösung abzentrifugiert, mit sterilem, redestilliertem Wasser gewaschen und in gleicher Weise zur neuen Züchtung weitergeführt, um damit dauernd Hefe zur Untersuchung zu erlangen. Nach mehreren Generationen, bis die Wachstumsgeschwindigkeit der Hefe keine Änderung mehr zeigte, waren die Hefen versuchsfertig. Wenn die Hefen nicht im Gebrauch waren, wurden sie in einer 0,75%-igen Monokaliumphosphatlösung suspendiert und im Eisschrank aufbewahrt. Ehe diese mehr oder weniger lange aufbewahrten Hefen zur Untersuchung kamen, wurden sie jedenfalls in der frischen Nährlösung mindestens einmal subkultiviert. Zur längeren Aufbewahrung wurden die Hefen in üblicher Weise in 10%-iger Zuckerlösung aufgeschwämmt und im Eisschrank gelagert.

Versuch. Dem Kulturkolben mit 1 *l* gereinigter Nährlösung wurden 10, 100 oder 1000 γ Eisen zugesetzt, und zwar in Form von Mohrschem Salz. Jeder Kolben wurde mit 0,5 g Hefe geimpft und 43 bis 44 Studen lang in gleicher Weise wie bei

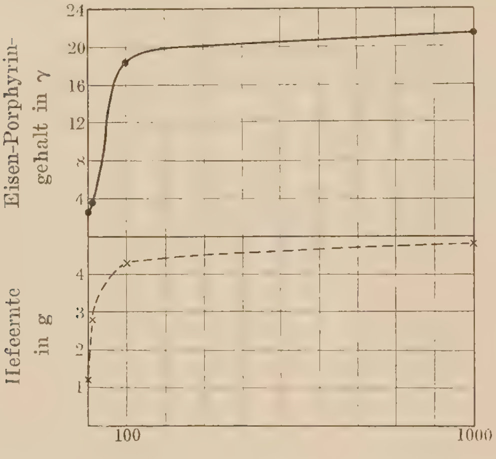
TABELLE IV.

Einfluss des Eisens auf den Eisen-Porphyringehalt der Hefe

Eisenzusatz pro Liter in γ	Hefeernte trocken in g	Eisen-Porphyrin		Porphyrin-Fe : Gesamt-Fe.
		für ganze Ernte in γ	für 1 g Trockenhefe in γ	
0	1,0	2	2	
	1,3	3	2,5	
	1,1	2	2	
	1,2	4	3,5	
	1,2	2,5	2	
Durchschnitt	1,2	3	2,5	(1 : 9)
10	3,6	7	2	
	2,5	12	5	
	2,4	8	3,5	
Durchschnitt	2,8	9	3,5	1 : 15
100	4,9	77,5	16	
	4,1	92	22,5	
	4,1	73,5	18	
	4,2	73	17,5	
Durchschnitt	4,3	79	18,5	1 : 15
1000	5,1	116	22,5	
	4,9	104	20,5	
	4,5	99	22	
Durchschnitt	4,8	106	21,5	1 : 110

Fig. 2.

Erntegwicht und Eisen-Porphyringehalt der Hefe beim Eisenzusatz in verschiedenen Konzentrationen.



Eisenzusatz in γ.

Subkultivierung gezüchtet. Da die Luftstrommenge für die Wachstumsgeschwindigkeit und für andere biologischen Eigenschaften der Hefe eine wichtige Bedeutung hat, wurde sie jedesmal um 50 *l* pro Stunde reguliert. Nach der Bebrütungszeit wurden sowohl die in der Nährlösung suspendierten als die an der Oberfläche sich aufschwemmenden Zellen als Versuchsmaterial geerntet. Das Hefematerial wurde durch Zentrifugieren von der Nährlösung getrennt, darauf in seiner Gesamtheit zweimal auf der Zentrifuge mit Aqua dest. gewaschen und auf der Nutsche scharf abgepresst. Nach dem Abwiegen der Gesamternte wurde ein Teil derselben zur Bestimmung der Trockensubstanzmenge verwendet, während mit dem Rest die Eisen-Porphyrinanalyse ausgeführt wurde. Im Falle einer auftretenden Infektion wurde der gesamte Versuch verworfen.

Die Resultate sind in Tabelle IV gezeigt.

Wie aus der Tabelle und Fig. 2 ersichtlich ist, steigt die Hefeernte von 1,2 bis 4,8 g, entsprechend den Zusätzen des Eisens von 0 bis 1000 γ; und zwar steigt die Kurve anfangs steil an, um dann langsam zur Horizontale umzubiegen.

Auch der Gehalt an Eisen-Porphyrin zeigt einen merklichen Sprung bei den Versuchen von 10 γ bis 100 γ Eisenzusatz, ungefähr den gleichen Wert wie bei Originalhefen erreichend (vgl. Tabelle III).

Die Analyse des Eisengehaltes der geernteten Hefe erwies sich, dass die Hefezellen das zugesetzte Eisen der Nährlösung restlos aufgenommen hatten. In der letzten Kolumne der Tabelle sind die Verhältnisse des Porphyrin-Eisens zum gespeicherten Gesamt-Eisen angegeben, wobei angenommen ist, dass in der gereinigten Nährlösung noch 2 γ Eisen als die letzte Verunreinigung unentfernt enthalten bleibt.

Das Resultat spricht dafür, dass eine minimale Menge des Eisen-Porphyrins zum Wachstum von Hefezellen unentbehrlich ist, und dass das Eisen als ein Aufbaumaterial schon in geringer Konzentration die Bildung des Eisen-Porphyrins beträchtlich stimuliert. Ist nur eine geringe Menge Eisen der Nährlösung zugefügt, nimmt der % Gehalt der geernteten Hefe an Eisen-Porphyrin wegen des viel rascheren Wachstums der Hefe nicht bedeutend zu, und erst beim Zusatz einer grösseren Menge Eisens wird die Hefe reich an Eisen-Porphyrin.

Es hat sich also gezeigt, dass unter meinen Versuchsbedingungen 100 γ Eisenzusatz pro 1 *l* Nährlösung zur maximalen Entwicklung Eisen-porphyrins notwendig und hinreichend ist.

3. *Einfluss des Kupfers auf die Entwicklung und den Eisen-Porphyringehalt der Hefe*

Versuch. Derselben Nährlösung wie im vorigen Versuche wurde 100 γ Eisen für 1 *l* zugesetzt und ihr dann die verschiedenen Mengen Kupfer in Form von Kupfersulfat zugefügt. Die sonstigen Arbeitsweisen waren genau wie beim vorigen Versuche.

Die Ergebnisse sind in Tabelle V angegeben. Die prozentualen Zunahmen der Hefeernte und der Eisen-Porphyrinmenge wurden in Fig. 3 graphisch gezeigt.

Aus der Tabelle und Figur ist zu ersehen: Der Zusatz des Kupfers in Gegenwart von Eisen erhöht die Eisen-Porphyrin-produktion in bedeutendem Masse; etwa 10 γ Kupfer pro 1 *l* Nährlösung übt eine optimale Wirkung aus. Bei höheren Kupferzugaben sinkt die Eisen-Porphyrinmenge der Hefe wieder ab. Das Kupfer erhöht zugleich die Trockensubstanzproduktion in niedrigeren Konzentrationen, indem höhere Gaben eine geringe Hemmung hervorrufen.

4. *Spektroskopische Untersuchung der Hefe.*

Entsprechend dem niedrigen Gehalt an Eisen-Porphyrin, sah die aus der Eisenfreikultur gewonnene Hefe krankhaft weiss aus; die spektroskopische Untersuchung dieser Hefe lehrt,

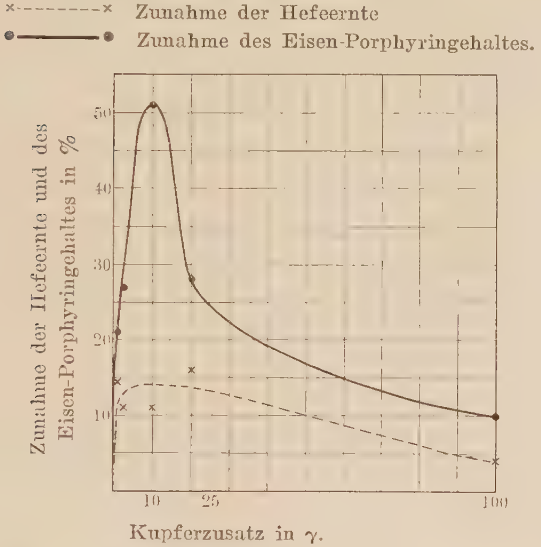
TABELLE V.

Einfluss des Kupferzusatzes auf den Eisen-Porphyringehalt der Hefe:

Kupferzusatz pro Liter in γ	Versuch Nr.	0	1	2.5	10	25	100
Hefeernte in g	(1)	5,0	6,1		5,6		
	(2)	4,8	5,3	5,0	5,5		
	(2)	4,6	5,0	5,3	4,9		
	(4)	4,4		5,1		5,5	4,2
	(5)	4,4				5,1	4,9
	Durchschnitt	4,6	5,5	5,1	5,1	5,3	4,6
Torales Eisenporphyrin für gesamte Hefeernte in γ	(1)	84	120		157		
	(2)	96	125	109	155		
	(3)	74	108	130	131		
	(4)	87		125		131	92
	(5)	97				149	118
	Durchschnitt	88	118	121	147	140	105
Eisen-Porphyrin für 1 g Trockensubstanz in γ	(1)	17	19.5		28		
	(2)	20	24	22	28		
	(3)	16,5	21,5	24,5	27		
	(4)	20		24,5		24	22
	(5)	22			29	29,5	24
	Durchschnitt	19	22	23,5	28	26,5	23

Fig. 3.

Zunahme der Hefeernte und des Eisen-Porphyringehalts durch Kupferzusatz zu eisenhaltiger Nährlösung.



dass der Gehalt der Hefe an Cytochrom ausserordentlich herabgesetzt war. Nur schwache Cytochrombänder b und c liessen sich erst in ziemlich dicker Schicht erkennen. Die Hefe aus der eisenhaltigen Nährflüssigkeit zeigte dagegen ein deutliches Cytochromspektrum; c-Streifen war in seiner Intensität am ausgezeichnetsten, b-Streifen etwas schwächer als c, und neben beiden scharfen Streifen war der schwache d-Streifen sichtbar. Alle Streifen nahmen mit steigender Eisenkonzentration der Nährlösung stark an Intensität zu. Aber es ist bemerkenswert, dass beim Zusatz von Eisen allein der Absorptionsstreifen von Cytochrom a spektroskopisch nicht nachgewiesen werden konnte. Erst nach dem Kupferzusatz trat der Absorptionsstreifen auf 604 mμ auf, und pararell zur Konzentration des Kupfers stieg die Intensität des roten Bande erheblich an. Es ist also festgestellt, dass die beiden Elemente Eisen und Kupfer zur Vervollständigung des typischen, vierbandigen Cytochroms notwendig sind. Bei zu hohen Gaben an Kupfer neigten die beiden grünen Bänder b und c zur geringen Intensitätsverminderung, wobei im einigen Fällen noch ein kontinuierlicher, verwaschener Schatten in der Gegend von grün bis Blau auftrat.

Auch das spektroskopische Bild des Pyridinextraktes der Hefe veränderte sich je nach der Art der Nährlösung. Die eisenfrei gezüchtete Hefe lieferte fast farblosen Pyridinextrakt, der nach Zusatz kleiner Menge Natriumhydrosulfits oder Hydrazins kaum erkennbares Hämochromogenspktrum aufwies. In dem Pyridinextrakte der auf der eisenhaltigen und kupferfreien Nährlösung gezüchteten Hefe, die reich an Eisen-Porphyrin war, wurden natürlich die gewöhnlichen Hämochromogenstreifen deutlich erkannt, aber der akzessorische Streifen auf 586 mμ nicht. Der letzte ging durchaus mit dem Cytochrom-a-Streifen parallel; es ist demnach mit grosser Wahrscheinlichkeit anzunehmen dass, wie schon von mehreren Forschern vermutet worden ist, (Anson, Mirsky) der Streifen auf 586 mμ, welcher in Pyridinextrakt aus Hefe auftritt, auf die a-Komponent des Cytochroms zurückzuführen ist.

Ergänzend sei hier angeführt, dass auch ein Unterschied in der Farbe der Hefe auffallend war; die Hefemasse aus der Nährlösung mit Eisen allein färbte sich rötlich, während die Hefemasse aus der Nährlösung mit Eisen und Kupfer gelblich aussah.

Im Anschluss an die bisher beschriebenen Untersuchungen bin ich auf die Frage eingegangen, inwieweit sich der Stoffwechseltypus oder die andere biochemische Beschaffenheit der Hefe auf die Veränderung des Eisen-Porphyringehaltes und des Cytochromspektrums bezieht.

III. Einfluss von Eisen und Kupfer auf die Atmungsfähigkeit und die katalytischen Eigenschaften der Hefezellen.

Zuerst habe ich Atmungs- und Gärungsgrösse der verschieden genährten Hefen untersucht. Im Anschluss hieran sind auch die katalytischen Eigenschaften, wie Katalasewirkung, Indophenoloxydasewirkung und Entfärbungszeit des Methylenblaus, untersucht worden.

Als Versuchsmaterialien wurden aus folgenden dreierlei Nährlösungen gewonnene Hefen ausgewählt: 1. Eine von Eisen und Kupfer befreite Nährlösung, 2. Dieselbe mit pro Liter zugesetztem 100 γ Eisen. 3. Dieselbe mit pro Liter zugesetztem 100 γ Eisen und 10 γ Kupfer. Der Einfachheit halber sollen hiernach diese dreierlei Hefematerialien als 1. Fe-frei-Hefe, 2. Fe-Hefe, 3. Fe-Cu-Hefe genannt werden. Die Züchtungsbedingungen waren genau wie bei den vorangehenden Versuchen.

A. Gaswechsel der verschieden genährten Hefen.

1. *Methodik zur Messung des Gaswechsels der Hefe.*

Der Sauerstoffverbrauch und die Kohlensäureproduktion kamen durch die bekannten Barcroft-Warburg-Manometer zur Messung.

Das Hefematerial wurde in folgender Weise hergerichtet. Nach der bestimmten Züchtungszeit wurde die Hefe von der Nährlösung abzentrifugiert und mit 0,75%-iger Monokaliumphosphatlösung zweimal gut gewaschen. Das so gewonnene Material wurde wiederum in Phosphatlösung aufgeschwämmt bzw. damit entsprechend verdünnt. Von dieser Suspension wurde 1 ccm in die Atmungsgefässe gebracht, zu der nachher je nach dem bestimmten Zweck entweder 1 ccm reine Monokaliumphsphatlösung (0,75%) oder 1 ccm Monokaliumphosphat-Rohrzuckerlösung (5% Zucker in 0,75%-iger Monokaliumphosphatlösung) hinzugefügt wurde. Die Versuche mit Zucker fanden also in 2,5%-iger Zucker-Phosphatlösung statt. Das einzelne Versuchsgefäss enthielt in der Regel etwa 2 mg Hefetrockensubstanz.

Als Hauptversuche wurden Sauerstoffverbrauch ohne und mit Zucker, Kohlensäureproduktion mit Zucker in Luft und in Stickstoff gemessen. Alle Versuche wurden bei einer Temperatur von 28°C in einem Warburgschen Thermostaten ausgeführt. Nachdem das Manometer 15 Minuten lang bis zum Temperaturausgleich geschüttelt worden war, wurde die Ablesung der Manometerausschläge begonnen.

Zur Ermittlung der Fehlergrenze, welche durch Aufbewahrung der Hefe in Phosphatlösung verursacht werden kann, wurden die Stoffwechselversuche mit verschieden lang aufbewahrten Hefesuspensionen ausgeführt. Falls sie im Eisschrank aufbewahrt wurden, blieben die Stoffwechselwerte der Hefezellen einige Tage unverändert, während, falls sie in Brutschrank von 25°C gebracht wurden, schon nach 18 Stunden eine auffallende Umstimmung eintrat. Es ist noch zu bemerken, dass die Werte im Laufe der Versuchszeit auffallenderweise variieren, was auch während der Vorschüttelungszeit unvermeidlich eintreten kann. Es ist deshalb darauf zu achten, dass bei den jeweiligen Messungen die Dauer des Vorschüttelns möglichst genau abgemessen wird.

2. Atmungs- und Gärungsversuche.

Die vergleichenden Versuche mit vorher angeführten Hefematerialien ergaben die in Tabelle VI und in Fig. 4 zusammengestellten Resultate.

Zum Verständnis der Versuchstabelle ist zu sagen, dass nach dem Vorschlag von Warburg folgende abgekürzte Bezeichnungen gewählt wurden: Q_{O_2} gleich Kubikmillimeter Sauerstoff, $Q^{O_2}_{CO_2}$ gleich Kubikmillimeter Gesamtkohlensäure, gemessen in Luft, und $Q^{N_2}_{CO_2}$ gleich Kubikmillimeter Kohlensäure, gemessen in Stickstoff, pro Milligramm Trockensubstanz und Stunde. Alle Werte wurden auf die ersten 30 Minuten der Versuchszeit bezogen. Ich habe auch den "scheinbaren respiratorischen Quotienten", den Wert $|Q^{O_2}_{CO_2}/Q_{O_2}|$ berechnet und gleichfalls in der Tabelle gezeigt.

Aus der Tabelle kann ersehen werden:

1. Vergleicht man die Mittel der Q_{O_2}-Werte ohne Zucker zwischen dreierlei Hefen, so findet man, dass der Wert für Fe-Hefe um 35%, für Fe-Cu-Hefe um 70% höher ist als für Fe-frei-Hefe.

2. Noch auffallender ist der Unterschied, falls die Atmungsgrösse der Hefe in Zucker-Phosphatlösung gemessen wird. In diesem Falle liegt das Mittel der Q_{O_2}-Werte für Fe-Hefe um

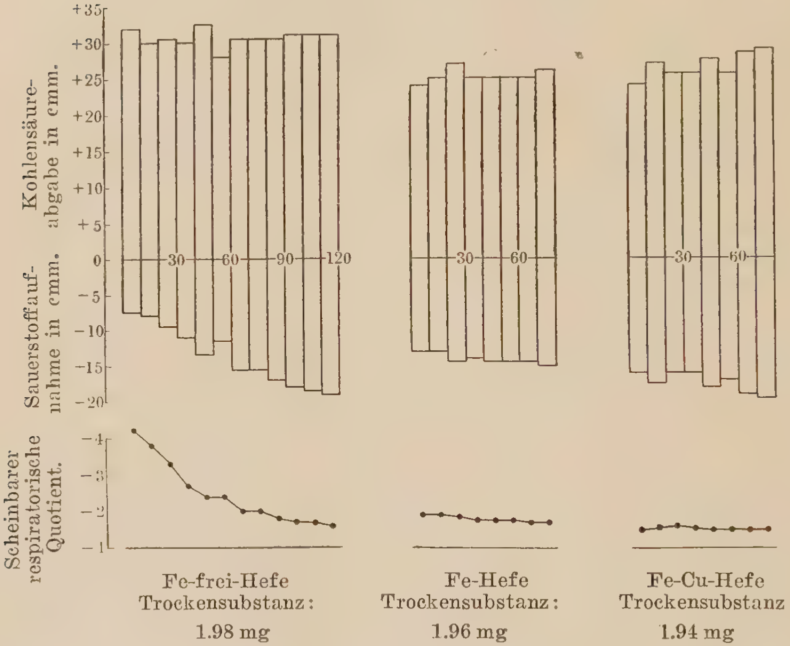
Tabelle VI.

Sauerstoffverbrauch, Kohlensäureproduktion im aeroben und anaeroben Zustand.

Hefematerial		Fe-frei-Hefe	Fe-Hefe	Fe-Cu-Hefe
Q_{O_2} ohne Zucker	1	− 20	− 29	− 45
	2	− 21	− 22	− 25
	3	− 25	− 35	− 42
	4		− 28	− 36
	5		− 39	− 37
	Durchschnitt	− 22	− 30	− 37
Q_{O_2} mit Zucker	1	− 27	− 44	− 60
	2	− 25	− 40	− 43
	3	− 22	− 48	− 52
	4	− 20	− 42	− 51
	5		− 45	− 55
	Durchschnitt	− 23	− 44	− 52
$Q^{O_2}_{CO_2}$	1	+ 85	+ 94	+ 95
	2	+ 93	+ 63	+ 52
	3	+ 91	+ 75	+ 76
	4	+ 81	+ 78	+ 79
	5		+ 81	+ 74
	Durchschnitt	+ 87	+ 78	+ 75
$Q^{N_2}_{CO_2}$	1	+106	+121	+155
	2	+102	+101	+ 91
	3	+101	+100	+111
	4		+123	+130
	5		+134	+122
	Durchschnitt	+103	+116	+122
Scheinbarer respiratorischer Quotient	1	3,2	2,1	1,6
	2	3,7	1,6	1,2
	3	4,2	1,6	1,5
	4	4,1	1,9	1,5
	5		1,8	1,3
	Durchschnitt	3,8	1,8	1,4

Fig. 4.

Sauerstoffverbrauch, aerobe Kohlensäureproduktion und scheinbarer respiratorische Quotient der verschieden genährten Hefen, gemessen und berechnet für je 10 Minuten.



90%, für Fe-Cu-Hefe um 125% höher als für Fe-frei-Hefe.

3. Im Gegensatz zu ansteigenden Atmungsfähigkeiten weisen die Fe- und Fe-Cu-Hefen durchschnittlich etwas niedrigere $Q^{O_2}_{CO_2}$-Werte auf als die Fe-frei-Hefen. Wenn man hier annimmt, dass die Atmungskohlensäure mit der Sauerstoffatmung parallel geht, so ist aus den Versuchsergebnissen zu schliessen, dass die aerobe Gärungsgeschwindigkeit der stärker atmenden Fe- und Fe-Cu-Hefe viel kleiner sein muss als die der schwächer atmenden Fe-frei-Hefe.

4. Der kleinere Sauerstoffverbrauch und die grössere Kohlensäureproduktion bei den Fe-frei-Hefen liefern selbstverständlich den höheren "scheinbaren respiratorischen Quotienten". Das gleiche trifft auch auf Fe-Hefe und Fe-Cu-Hefe

zu. Entsprechend der grösseren Atmungsgrösse der Fe-Cu-Hefe ist der "scheinbare respiratorische Quotient" bei der Fe-Cu-Hefe etwas kleiner als bei der Fe-Hefe.

5. Trotz des scharfen Unterschieds in bezug auf die Atmung und die aerobe Gärung zeigt die anaerobe Gärungsgrösse zwischen dreierlei Hefematerialien relativ geringe Unterschiede. Der durchschnittliche $Q_{CO_2}^{N_2}$-Wert für Fe- sowie Fe-Cu-Hefe ist ein wenig höher als der Wert für Fe-frei-Hefe.

6. Wenn man für einzelne Versuchshefen den $Q_{CO_2}^{O_2}$-Wert mit dem $Q_{CO_2}^{N_2}$-Wert vergleicht, so sieht man, dass die Einschränkung der Gärung durch die Sauerstoffatmung—sogenannte Pasteursche Reaktion—im Falle der Fe-Cu-Hefen am deutlichsten auftritt; dann folgen die Fe-Hefen, während die Fe-frei-Hefen nur schwache Pasteursche Reaktion aufzeigen.

Im Jahre 1925 hat Meyerhof in seiner bedeutsamen Arbeit über den Einfluss des Sauerstoffs auf die alkoholische Gärung der Hefe gezeigt, dass eine deutliche Verschiedenheit zwischen den Presshefen und den Brauereihefen in bezug auf den Stoffwechsel und besonders in bezug auf die Pasteursche Reaktion besteht. Die Atmungsgeschwindigkeit von Presshefe, gemessen in Zucker-Phosphatlösung, ist im Vergleich mit Brauereihefen beträchtlich gross. Auch die Einschränkung der Gärung durch die Atmung ist bei Presshefe viel deutlicher als bei Brauereihefen. Nach steigendem "scheinbaren respiratorischen Quotienten" geordnet ergab sich für die wichtigsten Hefearten die Reihenfolge: Torula (1,1), Bäckerhefe (2 bis 3), obergärige Bierhefe (6 bis 10), untergärige Bierhefe (10 bis 30).

Im Zusammenhang mit dieser von Meyerhof entdeckten Tatsache ist mein Ergebnis von grossem Interesse; das Verhalten der Fe-frei-Hefe ist mit der obergärigen Bierhefe zu analogisieren, dagegen lässt sich der Stoffwechseltypus der Fe-Hefe sowie der Fe-Cu-Hefe mit der wilden Hefe, Torula vergleichen.

Hier sei noch auf die Umstimmung des Stoffwechsels der Fe-frei-Hefe hingewiesen. Wie in der Figur dargestellt, nimmt die Atmung der Fe-frei-Hefe in Zucker-Phosphatlösung im Laufe des Atmungsversuches dauernd zu; die Kohlensäureerzeugung steigt indessen nicht an. Demzufolge fällt der "scheinbare respiratorische Quotient" mehr und mehr ab und nähert sich dem der Fe-Hefe oder der Fe-Cu-Hefe. Die Fe-frei-Hefe verhält sich also genau so, wie nach Meyerhof die obergärige Bierhefe in der Zucker-Phosphatlösung eine solche Umstimmung erfährt. Natürlich kann man über den Mechanismus dieser Umstimmung von Fe-frei-Hefe nicht sichereres sagen, als dass er noch einer eingehenderen experimentellen Erforschung bedarf.

B. Katalytische Eigenschaften der verschieden genährten Hefen.

1. *Methodik zur Messung der katalytischen Eigenschaften der Hefe.*

1. *Bestimmuugsmethode der Katalasewirkung.* Die Katalasestärke der Hefe kam nach der Vorschrift von Fujita und Kodama (1931) mit Hilfe des Barcroft-Warburg-Manometer zur Messung.

Da die Katalasewirkung der Hefezellen äusserst veränderlich ist, und wenn die Hefe von der Nährlösung abgetrennt und in Phosphatlösung suspendiert wird, die Katalasestärke in kurzer Zeit erheblich ansteigt, so ist es unmöglich, die Katalasewirkung der Hefe ohne besondere Vorbehandlung exakt zu messen.

Nach einigen Versuchen erwies es sich als zweckmässig, die Hefe in der mit Toluol gesättigten Phosphatlösung zu suspendieren und dann zur Messung zu bringen, da im Gegenwart von Toluol die Katalasestärke der Hefe ziemlich konstant blieb. Die von der Nährlösung abzentrifugierten Hefezellen wurden in der m/20 Phosphatpufferlösung von pH=7,4, die zuvor mit einigen Tropfen Toluol durchgemischt war, gleichmässig auf eine geeignete Konzentration aufgeschwämmt.

Die Messungen wurden mittels kegelförmiger Gefässe bei einer Temperatur von 28°C ausgeführt. Die initiale Konzentration des Wasserstoffperoxyds war 0,01 m. Als Mass für die Katalasestärke diente der "Katalasequotient" $Q_{Kat.}$ nach Fujita und Kodama, welches die Kubikmillimeter Sauerstoffablassung in 30 Minuten pro Milligramm Trockensubstanz der Hefe repräsentiert. Die gemessenen Werte wurden jeweils in Rücksicht auf die Atmung korrigiert.

2. Bestimmungsmethode der Indophenoloxydasewirkung. Zur quantitativen Bestimmung der Indophenoloxydasewirkung der Hefe wurde das Verfahren von Keilin (1929) in etwas modifizierter Weise angewendet.

Die Hefe wurde von der Nährlösung abzentrifugiert, auf Zentrifuge mit m/20 Phosphatpuffer von pH 7,4 zweimal gewaschen und wiederum in Phosphatpuffer auf die Konzentration von rund 10% aufgeschwämmt. Nun wurde die Suspension in Wasserbad von 52°C 1,5 Stunden lang erhitzt, wodurch das temperaturempfindlichere, die Oxydasereaktion beeinträchtigende Dehydrasesystem der Zelle nahezu vollkommen vernichtet wurde. Die Messungen wurden bei 28°C wieder mit Hilfe der Barcroft-Warburg-Manometer mit kegelförmigen Atmungsgefässen ausgeführt.

Hauptraum:	1 ccm wie oben vorbehandelte Hefesuspension + 1 ccm m/20 Phosphatpuffer pH = 7,4.
Einsatz:	0,5 ccm 10%-ige Kalilauge.
Birne:	0,2 ccm 1%-ige wässerige Lösung von p-Phenylendiamin.

Die Gefässe wurden mit den Manometern verbunden und im Thermostaten geschüttelt. Nach Temperaturausgleich wurde der Inhalt der Birne in den Hauptraum eingekippt und nach 30 Minuten der Manometerausschlag abgelesen. Die absorbierte Sauerstoffmenge in Kubikmillimeter pro Milligramm Trockensubstanz und Stunde soll durch die Bezeichnung $Q_{Ind.}$ gezeigt werden.

3. Bestimmung der Entfärbungszeit von Methylenblau. Der Versuch geschah durch das wohlbekannte Verfahren von Thunberg, wobei das von Hirade (1934) modifizierte Thunberg Rohr gebraucht wurde. Die Hefe wurde nach Waschen mit m/20 Phosphatpufferlösung von pH 7,4 in gleicher Phosphatlösung aufgeschwämmt. Die Trockensubstanzmenge in der Suspension war 20 bis 28 mg pro 1 ccm. In einen Schenkel des Thunberg Rohrs wurde 1 ccm der Hefesuspension gebracht, zu der entweder 1 ccm reiner Phosphatpufferlösung oder 1 ccm Glukose-Phosphatlösung (10% Glukose in Phosphatpuffer) hinzugefügt wurde; in dem anderen Schenkel wurde 1 ccm 0,02%-iger Methylenblaulösung aufgenommen. Alsdann wurde das Rohr mit einer Gaedeschen Vakuumpumpe verbunden und 1,5 Minuten lang unter Schütteln evakuiert.

Die Versuchstemperatur war auch hier 28°C. Nachdem das Rohr einige Minuten in dem Thermostaten geschüttelt wurde, wurde der Inhalt der beiden Schenkel durch Umstülpen des Rohrs vermischt und der Zeitdauer bis zur völligen Entfärbung beobachtet. Um die Vergleichung der einzelnen Versuchsdaten zu erleichtern, habe ich die Reduktionszeit in jedem Falle für 25 mg Trockensubstanzmenge umgerechnet.

2. *Versuchsergebnisse.*

Die erhaltenen Resultate sind in Tabelle VII aufgeführt. Wie daraus hervorgeht, nimmt der Gehalt der geernteten Hefe sowohl an Katalase als an Indophenoloxydase durch den Zusatz von Eisen zu der Nährlösung bedeutend zu. Der mittelere $Q_{Kat.}$- wie $Q_{Ind.}$-Wert der Fe-Hefe ist rund 6 mal so gross wie der der Fe-frei-Hefe. Diese Ergebnisse sprechen dafür, dass das Eisen ein speziell bei Katalase- sowie Indophenoloxydasebildung der Zelle notwendiges Element ist, und dass die Wirkungsweise der beiden Fermente, wie bisher vermutet worden ist, eine Eisenkatalyse sei.

Wenn man bezüglich der Indophenoloxydase die Fe-Hefe und die Fe-Cu-Hefe miteinander vergleicht, so sieht man eine bemerkenswerte Verschiedenheit zwischen den beiden. Die Indophenoloxydase der Fe-Cu-Hefe ist stets auffallend stärker als die der Fe-Hefe. Auch durch den Versuch mit Nadi-Reagens liess sich dies leicht erkennen. Cohen und Elvehjem (1934) beobachteten, dass sich die a-Komponente des Cytochroms in

TABELLE VII.

Katalasewirkung, Indophenoloxydasewirkung und Entfärbungszeit von Methylenblau.

Hefematerial		Fe-frei-Hefe	Fe-Hefe	Fe-Cu-Hefe
$Q_{Kat.}$	(1)			
	(2)	+57	+282	+222
	(3)	+41	+484	+238
	(4)	+26	+166	+236
	(5)	+43	+186	+369
	(6)		+235	
	Durchschnitt	+42	+271	+279
$Q_{Ind.}$	(1)	− 0,5	− 3,0	− 6,0
	(2)	− 0,5	− 2,4	− 6,0
	(3)	− 0,5	− 2,6	− 6,8
	(4)	− 0,2		
	(5)	− 0,3	− 2,2	− 5,4
	Durchschnitt	− 0,4	− 2,6	− 6,0
Entfärbungszeit von Methylenblau, ohne Zucker.	(1)		11′30″	10′40″
	(2)		21′	21′30″
	(3)	30′	17′	18′
	(4)	20′30″	11′40″	13′
	(5)	28′30″		
	Durchschnitt	26′	15′20″	16′
Entfärbungszeit von Methylenblau, mit Zucker	(1)		11′	10′40″
	(2)		12′	15′30″
	(3)	18′	11′30″	14′
	(4)	15′30″	7′20″	9′
	(5)	18′40″		
	Durchschnitt	17′20″	10′30″	12′20″

Herzmuskel und Lebergewebe der Ratten bei hochgradiger Anaemie ausserordentlich reduzierte und dabei die Nadi-Reaktion nur schwach war. Die Fütterung mit Kupfer oder Kupfer und Eisen vermehrte dabei den Oxydasegehalt merklich, Eisen allein war aber vollkommen erfolglos. Mein Befund lehrt also in Übereinstimmung mit ihren Resultaten, dass das Kupfer die

Bildung der Indophenoloxydase stark stimuliert, und dabei die Vermehrung der a-Komponente des Cytochroms immer beobachtet wird. Aber die Frage, ob das Kupfer zur Indophenoloxydasebildung unentbehrlich sei, bleibt durch diesen Versuch noch ungelöst, da die Kupferspur, die in der Nährlösung noch unentfernt zurückbleibt, auf die Bildung der Oxydase einen ausschlaggebenden Einfluss ausüben kann.

Die Katalasewirkung der Hefe scheint dagegen durch den Kupferzusatz nur in unbedeutendem Masse beeinflusst zu werden.

V. ZUSAMMENFASSUNG.

1. Es wurde eine Bestimmungsmethode des mit Pyridin extrahierbaren Eisen-Porphyrins der Hefe angegeben.

2. Wenn die Hefe auf der von Eisen befreiten Nährflüssigkeit kultiviert ist, nimmt der Gehalt an Eisen-Porphyrin stark ab. Eisenzusatz zu Nährlösung resultiert eine merkliche Zunahme an Eisen-Porphyringehalt. Gleichzeitiger Zusatz der geeigneten Menge Kupfers mit Eisen erhöht die Eisen-Porphyringehalt der Hefe noch weiter, aber zu hohe Kupfergaben rufen eine geringe Verminderung des Eisen-Porphyrins hervor.

3. Die Hefe, welche aus der eisenfreien Kultur gewonnen ist, ist durch kleinere Atmungsgrösse, grössere aerobe Kohlensäureproduktion, weniger ausgeprägte Pasteursche Reaktion, niedrigeren Gehalt an Katalase wie Indophenoloxydase gekennzeichnet. Auch das Reduktionsvermögen des Methylenblaus ist etwas herabgesetzt.

4. Die Atmungsgrösse nimmt durch Eisenzusatz zur Nährlösung beträchtlich zu, während die aerobe Kohlensäureproduktion ein wenig abnimmt; die Pasteursche Reaktion tritt viel stärker auf als bei der eisenfrei gezüchteten Hefe. Sehr auffallend ist die Verstärkung der Katalase- und der Indophenoloxydasewirkung.

5. Durch Zugabe von Kupfer zu der eisenhaltigen Nährlösung wird die Indophenoloxydasewirkung stark gesteigert, während die anderen Eigenschaften, wie Atmungsgrösse, Kohlensäureproduktion, Katalasewirkung und Methylenblauentfärbungs-

zeit, wenig beeinflusst werden.

6. Die aus der Eisenfreikultur gewonnene Hefe zeigt nur schwache Cytochromstreifen b und c. Durch Zusatz von Eisen werden die beiden Streifen intensiv, jedoch kein Streifen im Rot. Erst nach dem Kupferzusatz zu der eisenhaltigen Nährlösung gewinnt man die Hefe, welche neben den Streifen b und c den Cytochrom-a-Streifen aufweist.

Es ist mir ein Bedürfnis, Herrn Prof. Dr. S. Kakiuchi für seine freundlichen Ratschläge bei dieser Arbeit meinen besten Dank auszusprechen.

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SUR LE METABOLISME DE LA CHOLESTERINE CHEZ LES COBAYES INOCULES AVEC DES BACILLES TUBERCULEUX.

I. Sur le taux des cholestérines libres et éthérifiées dans le sang total du cobaye normal et du cobaye tuberculeux.

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Des nombreux auteurs ont déja publié plusieurs travaux sur le changement des lipoides dans le serum des personnes tuberculeuses et voulu établir des relations entre la tuberculose et la valeur de la cholestérine dans le serum. Mais les résultats obtenus sont tout à fait contradictoires. Pour acquérir une conception plus biologique, il est nécessaire de faire une série d'expériences sur un grand nombres de sujets, sur les relations entre le taux de ces éléments et l'effet pathologique. Alors j'ai entrepris de prélever le sang des cobayes et de les disséquer tout de suite après. Cette dissection a été décrite selon la méthode de Monsieur le Professeur S. Sato (1934) telle qu'elle est décrite à la page suivante. En premier lieu, j'ai voulu déterminer le taux de la cholestérine libre et éthérifiée dans le sang total en le comparant avec le résultat de la dissection Tel est l'objet principal du présent travail.

Marche des expériences.

Les animaux.

Pour mes expériences, je me suis servi de 50 cobayes pesant chacun de 350 gr. à 450 gr. La nourriture était constituée pour chaque cobaye par un mélange de 50 gr *d'okara*, 7.5 gr de carotte, 12.5 gr d'orge et 25 gr de choux comme légumes, et je les avais gavés chaque jour matin et soir. L'expérience a été commencée en laboratoire 3 semaines après l'achat. Cependant j'ai constaté que tous les cobayes utilisés étaient normaux c'est à dire négatifs envers la tuberculino-reaction de Römer-Joseph (1909). Les cobayes sont partagés en 5 groupes principaux, à savoir:

1^er^ groupe: les témoins.

2$^{\text{ème}}$ groupe: ceux qui ont reçu une 0.01 mg d'injection sous-cutanée d'émulsion de bacilles tuberculeux dans la région hypogastrique gauche.

3$^{\text{ème}}$ groupe: ceux qui ont reçu une 0.1 mg d'injection d'émulsion des bacilles tuberculeux.

4$^{\text{ème}}$ groupe: ceux qui ont reçu la même injection que la 3$^{\text{ème}}$ groupe. Ce groupe a été gracieusement donné par Monsieur le Professeur S. Sato à l'Institut des Maladies Infectieuses de l'Université Imperiale de Tôkyo.

5$^{\text{èm}}$ groupe: ceux qui ont reçu une 1.0 mg d'injection d'émulsion de bacilles tuberculeux.

L'expérience et la dissection furent exécutées au bout de 10 semaines, car le Professeur Sato déclare que les symptômes tuberculeux apparaissent entièrement à cette époque. Les résultats de la dissection ont été constatés par M. le Dr. Yanagisawa de l'Institute des Maladies Infectieuses de l'Université Impériale de Tôkyo.

L'EMULSION DE BACILLES TUBERCULEUX.

Les bacilles que j'ai utilisés ici sont proviennent du service de fabrication de la tuberculine de l'Institut des Maladies Infectieuses. Ces bacilles avaient été cultivés à 37°C pendant 3 à 4 semaines sur bouillon glycériné. Ils étaient recueillis et pressés sur le papier filtre stérilisé pour éliminer le plus possible la liquide qui les imprégnait. Alors ils étaient mesurés en poids défini et émulsionnés avec la solution physiologique de chlorure de sodium.

L'émulsion était diluée avec cette solution de manière que 0.5 cc. de l'émulsion contienne respectivement 1.0 mg. 0.1 mg et 0.01 mg de bacilles tuberculeux. Tous les traitements étaient aussi aseptiques que possible.

LA DETERMINATION DE LA CHOLÉSTÉLINE.

Le prélèvement de sang était fait par ponction au coeur. Le sang était toujours pris à jeun, entre 20 et 24 heures après l'alimentation. Pour empêcher le sang de coaguler, les haparines (Schering-Kahlbaum) ont été utilisés comme anticoagulants. Ils étaient employé au taux de 5 milli-grammes par 5 cc. de sang.

Le cholestérole dans le sang total était dosé selon la technique de Monsieur le Dr. Onizawa. (1928/29) Mais cette méthode ayant l'inconvénient de laisser des bacilles vivants, voici comment j'ai procédé: Pour extraire la cholestérine, 150 cc. d'un mélange d'acétone et d'alcool (à volume égal) est utilisé pour 5 cc. du sang total. J'ai extrait cette quantité de sang avec le rafraîchisseur reflux pendant 4 à 5 heures. Après avoir épuisé la graisse du sang par le mélange de solvant, j'ai fait le même traîtement selon la méthode du Dr. Onizawa.

RÉSULTATS EXPÉRIMENTAUX.

1. Dans le premier groupe des cobayes, les témoins, j'ai d'abord mesuré le taux de la cholestérine libre et éthérifiée dans le sang total des cobayes 3 semaines après leur vie de laboratioire et en même temps le poids corporel. Après 10 semaines, j'ai fait le même calcul sur les mêmes animaux.

J'ai montré dans les tableau I et II les chiffres obtenus pour des cobayes ainsi expérimentés.

1er Groupe

TAB. I.

L'augmentation du poids du corps du cobaye à l'état normal.

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Moyenne
	gr.	gr.	gr.	gr.	gr.	gr.	gr.
Avant le traitement	380	390	370	435	450	410	406
10 semaines après	600	520	540	625	625	590	584
différence	+220	+130	+170	+190	+175	+180	178

Tab. II.

La teneur des cholestérines libres et éthérifieés dans
le sang total des cobayes témoins
valenr exprimée en gr pour 100 cc. du sang total

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Moyenne
	%	%	%	%	%	%	%
Chol. total	0.069	0.077	0.065	0.058	0.068	0.089	0.071
Chol. libre	0.050	0.065	0.054	0.044	0.052	0.051	0.056
Chol. éthér.	0.019	0.012	0.011	0.014	0.016	0.018	0.015
libr/éthér.	2.6	5.4	4.9	3.2	3.3	2.8	3.7
	Après 10 semaines						
	%	%	%	%	%	%	%
Chol. total	0.068	0.091	0.055	0.060	0.088	0.073	0.073
Chol. libre	0.055	0.075	0.044	0.050	0.073	0.055	0.059
Chol. éthér.	0.014	0.016	0.011	0.010	0.015	0.018	0.014
libre/éthér.	3.9	4.7	3.9	5.0	6.4	4.7	4.2

Le tableau II montre qu'il n'y a pas de différence considérable dans le taux de la cholestérine avant et après 10 semaines chez les cobayes témoins.

2. Les résultats des expériences du $2^{ème}$ groupe conduites comme je l'ai indiqué plus haut, sont consignés dans le tableau suivant (Tableau III, IV et V).

$2^{ème}$ Groupe.

Tab. III.

L'augmentation du poips du corps du cobaye tuberculeux

	No. 31	No. 32	No. 33	No. 34	No. 35	No. 36	No. 37	No. 38	No. 39	No. 40	Moyenne
	gr	gr	gr	gr	gr	gr	gr	gr	gr	gr	gr
Le poids du corps avant l'expériance	380	360	340	380	380	350	380	370	400	340	362
Le poids du corps a la $1^{ème}$ semaine après la contagion.	520	480	400	mort	mort	mort	570	510	mort	410	482
La différence du poids du corps	+140	+120	+ 60				+190	+140		+ 70	+120

TAB. IV.

Le résultat de la dissection du cobaye tuberculeux

	No. 31	No. 32	No. 33	No. 34	No. 35	No. 36	No. 37	No. 38	No. 39	No. 40
L'ulcère local	—	—	—	manqué	manqué	manqué	—	—	manqué	—
Le ganglion lymphatique inguinal superficiel	+	++	+				+	++		++
Le ganglion lymphatique iliaque gauche	—	—	—				—	—		—
Le ganglion lymphatique inguinal superficiel droit	+	—	—				—	—		—
Le ganglion lymphatique iliaque droit	—	—	—				—	—		—
Le ganglion lymphatique axillaire gauche	—	—	—				—	—		—
Le ganglion lymphatique axillaire droit	—	—	—				—	—		—
Le ganglion lymphatique rétrosternal	—	—	—				—	—		—
Le ganglion lymphatique rétropéritoneal	—	—	—				—	—		—
Le ganglion lymphatique periveineux portae	—	—	—				—	—		—
Le ganglion lymphatique tracheobronchial	—	—	—				—	—		—
Le poumon	—	—	—				—	—		—
Le foie	—	—	—				—	—		—
La rate	—	—	—				—	—		—
Le poids de la rate	0.8 gr	0.6 gr	1.3 gr				0.7 gr	0.6 gr		0.4 gr

Les marques (+) et (—) indiquent le degré de l'altération tuberculeuse dans le ganlion lymphatique.

+	signifie le gonflement de la grandeur d'un grain de riz.
++	signifie la gonflement de la grandeur d'un petit pois.
+++	signifie de la grandeur d'un haricot.
++++	signifie de la grandeur d'une fève.

Dans les viscères.

+	signifie l'existence de 1–2 tubercules dans l'organe.
++	signifie l'existence d'un certain nombre de tubercules dans l'organe.

+ + + signifie que moins de la moitié de l'organe est atteint.
+ + + + signifie que plus de la moitié de l'organe est atteint.

Tab. V.

La teneur des cholestérines libres et éthérifiées dans le sang total du cobaye tuberculeux.

	No. 31 | No. 32 pris des duex	No. 33	No. 37 | No. 38 pris des deux	No. 39	Moyenne
	%		%		%
Cholestérole total	0.090	manqué	0.084	manqué	0.087
„ libre	0.079		0.071		0.075
„ éthérifié	0.011		0.013		0.012
libr/éthér.	7.1		5.5		6.3

Le tab. III montre moins d'augmentation du poids corporel des cobayes tuberculeux que de celui des témoin.

Le tab. IV. montre que les animaux ne présentent que peu d'altération tuberculeuse. Le tab. V n'indique aucune différence entre l'état normal et l'état pathologique.

3. On trouvera dans le tableau V, VI, VIII ci-dessous les résultats des expériences du 3ème groupe.

3ème Groupe.

Tab. VI.

L'augmentation dn poids du corps du cobaye tuberculeux.

	No. 21	No. 22	No. 23	No. 24	No. 25	No. 26	No. 27	No. 28	No. 29	No. 30	Moyenne
	gr	gr	gr	gr	gr	gr	gr	gr	gr	gr	gr
Le poids du corps avant l'expérience	340	340	270	340	320	340	mort	270	320	320	318
Le poide du corpe à la 10ème semaine après la contagion	mort	mort	mort	380	340	420	—	380	430	360	385
La différence	—	—	—	+ 40	+ 20	+ 80	—	+110	+110	+ 40	+ 67

TAB. VII.

Le résultat de la dissection du cobaye tuberculeux.

	No. 21	No. 22	No. 23	No. 24	No. 25	No. 26	No. 27	No. 28	No. 29	No. 30
l'ulcère local	mort	mort	mort	–	–	–	mort	–	–	–
Le ganglion inguinal superficiel gauche				++	–	+		+++	++	++
Le ganglion iliaque superficiel gauche				–	–	–		–	–	–
Le ganglion inguinal superficiel droit				+	+	–		++	+	–
Le ganglion iliaque droit				–	–	–		–	–	–
Le ganglion axillaire gauche				–	–	–		–	–	–
Le ganglion axillaire droit				–	–	–		–	–	–
Le ganglion retro-sternal				+	–	–		–	–	–
Le ganglion retro-peritoneal				–	–	–		–	–	–
Le ganglion peri-veineux portae				+	–	–		+?	+?	+
Le ganglion tracheo-bronchial				–	–	–		–	–	–
le poumon				–	–	–		–	–	–
le foie				–	–	–		–	–	–
le rate				++	–	–		–	–	++
le poide de la rate				2.1 gr	0.6 gr	0.6		0.9	0.9	2.3

TAB. VIII.

La teneur de cholestérines libres et éthérifiées dans le sang total du cobaye tuberculeux.

	No. 24	No. 25	No. 26	No. 28	No. 29	No. 30	Moyenne
		pris de deux		pris de deux			
		%		%			%
Cholestérole total	manqué	0.095		0.073		manqué	0.084
Cholestérole libre		0.080		0.059			0.070
Cholesterole éthärifié		0.015		0.014			0.015
libre/éthér.		5.3		4.2			4.7

Le tab. VI démontre que l'augmentation du poids corporel est très faible, ce que j'attribue au poids initial de moins de 350 grammes.

L'altération tuberculeuse contatée dans le tableau VII était la même que celle du tableau IV. Le tab. VIII n'indique aucune différence entre l'état normal et l'état pathologique.

4. Les résultats d'expériences du 4ème groupe sont réunis dans les tableaux IX, X et XI. suivants.

C'est le groupe des cobayes donnés par le Prof. Sato et M. Dr. Yanagisawa de l'Institut Impérial de Maladies Infectieuses. Ils les avait utilisés comme témoins pour la cure chimique des cobayes tuberculeux. Dans tous les cas des tab. X, VII et IV il n'y a presque aucune altération tuberculeuse. Mais j'ai constaté l'altération tuberculeuse dans la préparation histologique du ganglion lympatique gonflé. Le Dr. Yanagisawa a bien voulu examiner mon expérience et déclarer qu'il étaite de même avis. Le lecteur observera que le résultat du tab. X est identique à celui des tab. VII et IV.

5. Les cobayes du 5ème groupe ont donné les résultats que l'on trouve dans les tableaux suivants.

Le tableau XIV montre que la cholestéline libre augmente de 50% jusqu'à 14% et la cholestérine éthérifiée de 133.3% jusqu'à 0%.

4ème Groupe.

TAB. IX.

L'augmentation du poids du corps du cobaye tuberculeux.

	No. 51	No. 52	No. 53	No. 54	No. 55	No. 56	No. 57	No. 58	No. 59	No. 60	moyenne
	gr	gr	gr	gr	gr	gr	gr	gr	gr	gr	gr
Le poids du corps avant l'experience	405	477	437	450	380	455	405	410	390	377	410
le poids du corps à la 10ème semaine après l'infection	530	mort	515	560	510	555	515	525	520	600	536
la différence du poids du corps	+125		+78	+110	+130	+120	+110	+115	+130	+223	+126

Tab. X.

Le résultat de la dissection du cobaye tuberculeux.

	No. 51	No. 52	No. 53	No. 54	No. 55	No. 57	No. 58	No. 59	No. 59	No. 60
L'ulcère local	—	mort	—	—	—	—	—	—	—	—
le ganglion inguinal superficiel gauche	+		+ +	+	+	+	+	+	+	—
le ganglion iliaque gauche	—		—	—	—	—	—	—	—	—
Le ganglion inguinal superficiel dorit	+ +		+ +	+	+	+	+ +	+ +	+ +	+
Le ganglion iliaque droit	+		—	+	+	—	+	+	—	—
Le ganglion axillaire gauche	—		+	—	—	—	+	—	—	—
le ganglion axillaire droit	—		—	—	—	—	—	—	—	—
le ganglion retrosternal	—		+ +	—	—	—	—	—	—	—
le ganglion retroperitoneal	+		—	—	—	—	—	+	+	—
le ganglion periveineux portae	+		+ + +	—	—	—	—	—	—	—
le ganglion tracheobronchial	—		+ + +	—	—	—	—	—	—	—
le poumon	—		+ +	—	—	—	—	—	—	—
la foie	—		+ + +	—	—	—	—	—	+	—
le poids de la rate	0.7		1.7	0.5	0.6	0.4	0.5	0.5	0.5	0.7

Tab. XI.

La teneur des cholestérines libres et éthérifiées dans le sang total du cobaye tuberculeux.

	No. 51	No. 53	No. 54	No. 55	No. 56	No. 57	No. 58	No. 59	No. 60	moyenne
			pris de deux			pris de deux		pris de deux		
	%	%	%			%		%		%
Cholestérole	0.068	0.100	0.088		manqué	0.090		0.089		0.087
Cholestérole libre	0.045	0.088	0.073			0.077		0.071		0.071
Cholestérole éthérifié	0.023	0.012	0.015			0.013		0.017		0.016
libre/éther.	2.0	7.3	4.9			5.9		4.2		4.4

5ème Groups.

Tab. XII.

L'augmentation du poids du corps du cobaye tuberculeux.

	No. 81	No. 82	No. 83	No. 84	No. 85	No. 86	No. 87	No. 88	No. 89	No. 90	Moyenne
Le poids du corps avant l'expérience	385	340	390	430	370	420	385	380	415	390	388
le poids du corps à la 10 ème semaine après l'infection	455	500	mort	520	385	425	mort	mort	mort	585	485
la différence de poids du corps	+ 70	+160		+ 90	+ 15	+ 5				+195	+ 97

Tab. XIV.

La teneur des cholésterines libres et éthérifiées dans le sang total du cobaye tuberculeux

	No. 81	No. 82	No. 84	No. 85	No. 86	No. 88	No. 90	Moyenne
Cholestérole total	0.120	0.081	0.094	0.063	0.110	0.087	0.103	0.094
Cholestérole libre	0.085	0.066	0.073	0.051	0.078	0.068	0.089	0.074
Cholestérole éthérifié	0.035	0.015	0.021	0.012	0.032	0.019	0.014	0.022
libre/éthér	2.4	4.3	3.5	4.3	2.4	3.6	6.4	3.4

L'augmentation de la dernière est donc plus forte que celle de la première. Le cobaye No. 85 dans le tableau XIV est une exception: chez lui la valeur de la cholésterine libre et éthérifiée était au-dessous de la normale et la tuberculose des viscères était de la forme exsudative.

RÉSUMÉ

Je ne tirerai ici aucune conclusion, parce que je veux étudier plus en détail les sujets suivants.

(1) A quels éléments du sang est due l'augmentation de la cholestérine dans le sang total des cobayes tuberculeux? aux globules rouges ou au plasma?

(2) Au bout de combien de semaines commence-t-elle? Quel rapport a-t-elle avec l'apparition de la réaction tuberculine?

Tab. XIII.

Le résultat de la dissection du cobaye tuberculeux

	No. 81	No. 82	No. 83	No. 84	No. 85	No. 86	No. 87	No. 88	No. 89	No. 90
L'ulcère local	cm 3	cm 3	mort	cm 0.3	cm 3	–	mort	cm 2	mort	cm 1
le ganglion inguinal superficiel gauche	++++	++++		++++	+++	++++		++++		++++
le ganglion iliaque gauche	+	++		++	+	++		–		++
le ganglion inguinal superficiel droit	++++	++		+++	++	++		++		+++
le ganglion iliaque droit	++	+		–	–	–		–		–
le ganglion axillaire gauche	++	++		++	++	++		+		+++
le ganglion axillaire droit	+	+		++	++	++		–		+++
le ganglion retrosternal	+	++		++	–	–		–		+
le ganglion retroperitoneal	++	+++		++	+	++		++		+++
le ganglion periveineux portae	++	++++		+++	++	+++		++		++++
le ganglion tracheobronchial	++	++++		++++	+++	++		++		++++
le poumon	++++	+++		++++	++++	–		+		++
le foie	++++	++++		++++	++++	++		+++		+++
La rate	++++	++++		++++	++++	+++		+++		+++
le poids de la rate	6.6	6.8		4.0	25.2	4.6		2.7		1.5

(3) Dans quels organes observe-t-on l'augmentation et la diminution de la valeur de la cholestérine?

(4) Les deux formes de la tuberculose, productive et exsudative, ont-t-elles quelque rapport avec la valeur de la cholestérine dans le sang total?

je me bornerai à résumer comme suit mes expériences.

1. Il n'y a aucune différence entre les cobayes témoins et les cobayes tuberculeux, qui ont reçu une injection souscutanée de 0.01 mg et 0.1 mg d'émulsion de bacilles tuberculeux de virulence faible. Ils ont seulement une légère altération tuberculeuse du ganglion lymphatique régional après 10 semaines.

2. Les cholestérines libres et éthérifiées dans le sang total augmentent en moyenne d'environ 27% et 40% chez les cobayes tuberbuleux qui ont reçu en injection, l'émulsion de 1.0 mg de bacilles tuberculeux de virulence puissante. On constate une grave altération tuberculeuse de plusieurs ganglions lymphatiques et de plusieurs viscères dans la dissection après 10 semaines de contagion.

Ce m'est un devoir d'exprimer ma reconnaissance à M. le Professeur S. Kakiuchi de la Faculté de Médecine de l'Université Imperiale de Tokyo pour l'aide, qu'il n'a cessé de me prodiguer au cours des ces travaux. Je remercie égalemant M. le Professeur S. Sato et le Docteur K. Yanagisawa à l'Institut des Malades Infectieuse de l'Université Impériale de Tokyo qui m'ont procuré très gracieusement des matériaux précieux pour mes recherches.

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SOME POSSIBLE FACTORS CONCERNING THE DEVELOPMENT OF DENTAL CARIES.

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INTRODUCTION.

Dental Caries is the most widely distributed disease of mankind, peoples of all countries and all races being subjected to its ravage. It has been estimated that from 85 to 95 percent of the people of the civilized races suffer from it.

There are many factors which induce a tooth to develop caries, although the susceptibility to the disease fluctuates from time to time.

The position of the teeth, the structural peculiarities of the enamel and of the dentine, the character of the diet, the amount and composition of the saliva, the sort of bacterial flora, certain constitutional factors, and the degree of hygienic care of the mouth, are factors which have been considered as responsible for the development of caries.

Although the chemico-paratitic theory proposed by W. D. Miller (1885) to explain the development of dental caries is fairly satisfactory and is almost universally accepted at the present time, there remain still certain important phases of the problem to be explored. Experiments concerning these points are reported in the following pages.

I. THE SOLUBILITY OF HUMAN ENAMEL

The first experiment was to investigate the solubility of enamel by acidic substances under different conditions.

Experiments on enamel solubility have been conducted previously by H. P. Pickerill (1924) ; J. Head (1920) ; F. F. Kanthak (1932) ; J. R. McClelland (1926)., etc. Their methods lacked, however, generally quantitative treatments, and their results are hardly useful for exact discussion.

A. Material.

The enamel used in this experiment was all prepared myself from molars chosen from the same aged people and free from diseases of hard structure. The enamels were sectioned into moderate pieces (Ca 50 mg of weight), and special attention was paid to free them from dentine.

The pieces were then washed first in running water, followed by distilled water, and placed in alcohol for one hour ; then put for fifteen minutes in the ether and kept in a desiccator for over a day or until their weight became constant. Each piece of enamel was then carefully weighed. This washing and drying treatment was naturally repeated also before weighing after treatment with acids.

B. Method of Experiment.

Each piece of enamel was placed over glass wool in a small beaker with many holes in the bottom. A dozen beakers of this kind were again placed on a round table with many holes and the whole was immersed into 50. cc of different sorts of acidic solutions contained in a beaker of 100 cc. capacity (See Fig. 1). After the lapse of 12 hours the pieces were taken out and rinsed with distilled water and dried through alcohol and ether.

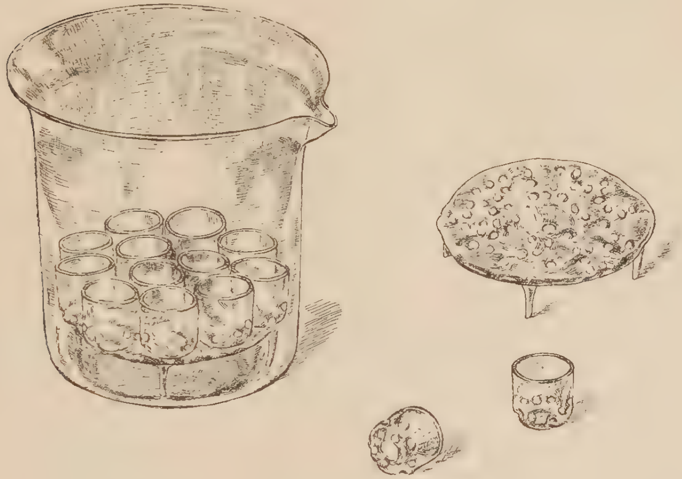
The difference of weight of each piece before and after the treatment was taken as the amount of enamel dissolved.

Reagents. The reagents used for the preparation of the buffer solution and each kind of acid used were all high grade Merck samples.

The pH value of the solution was determined colorimetrically.

Viscosity. The viscosity of the solution was determined by the use of Ostward's viscosimeter and expresed as relative viscosity.

Fig. 1.



C. *Results of Experiment.*

1. *The solubility of enamel by a solution different in pH value.*

The enamel used in this experiment and the following ones

Table I.

PH	Sort of buffer	Duration of experiment	Loss of weight
1.038	$\frac{N}{10}$ HCl	Ca 11 hours	entirely dissolved
2.0	Citrate-HCl	„ 53 „	„
3.0	„	„ 5 days	„
4.0	Acetate	„ 13 „	„
5.0	„	„ 34 „	„
5.57	Citric acid-NaOH	„ 10 „	27.28 %
5.97	„	„ „ „	12.99 %
6.33	„	„ „ „	5.03 %
6.77	Boric acid-borax	„ 50 „	none
7.94	„	„ „ „	„
8.98	„	„ „ „	„
6.67	Distilled water	„ „ „	„

consisted almost always of pieces taken from individuals twenty five years of age.

The solutions of different acidity were obtained by the use of citrate-HCl, acetate-acetic acid, citric acid-NaOH, and boric acid-borax.

As can be seen from table I enamel does not dissolve in a solution over 6.77 Ph value even after 50 days, while it dissolves quite readily when the Ph value is lowered under 4.

The solution at Ph 6.33 begins to dissolve enamel after prolonged application.

2. *The solubility of enamel in different sorts of fruit juice.* In this experiment the solubilities of enamel in the juice of lemon, citrus sinense, apple, grape, pear and vinegar were compared.

The Ph value of each solution was also determined. The result of the experiment is given in order of solubility.

TABLE II.

Sort of juice	PH	Percentage of enamel dissolved
Lemon	2.4	77.03 %
Vinegar	3.1	27.89 %
Citrus sinense	2.6	18.85 %
Apple	3.7	5.08 %
Grape	3.3	2.47 %
Pear	4.4	1.72 %
Grape of Italian Origin	3.5	1.45 %

The table shows that the solubility of enamel did not solely depend on the PH value of the solution. This suggests that other factors were also present in these cases.

I have, therefore, proceded to examine the effect of the viscosity of the solution and of titration acidity on the solubility of enamel.

3. *The effect of the viscosity of the solution of the solubility of enamel.*

Three sorts of solutions of the same acidity and of different viscosity were prepared by the mixture of 40. cc of 0.7 mol acetic acid with non, 5. cc, and 10. cc of glycerin and putting the whole of each mixture into 50. cc of distilled water.

The viscosities of these solutions were 1.073 1.397 and 1.832 respectively.

The solubilities of enamel in these solutions were as follows.

TABLE III.

Permanent Enamel (from persons 25 years old)

Viscosity	Solubility of enamel	PH
1.073	33.06 %	2.5
1.397	26.53 %	2.5
1.832	18.54 %	2.5

The table indicates that the more the solution is viscous, the less solubility results.

4. The effect of titrable acidity on the solubility of enamel.

99.05 cc. of 90 % lactic acid (Merk) were added to 141.98 cc of I. N. NaOH and diluted with water to 1. liter. The PH value of this solution was 3.1. By adequate dilution of this solution, solutions of different molar concentrations (0.5, 0.22, 0.1 and 0.05) were prepared. The viscosity of the solution was made the same (1.13) in all solutions by the addition of glycerin. Into this series of such solutions enamel from a person 25 years old was immersed for 12 hours 22°C. The result is shown in table 4.

TABLE IV.

concentration in mol	0.5	0.22	0.1	0.05
percentage of solubility	83.54	59.51	36.63	24.18

As is clear from the table, enamel dissolves much greater in the more concentrated lactic acid than in its weaker solutions of the same hydrogen ion concentration.

5. The effect of titration and PH value of lactic acid on the solubility of enamel.

Three kinds of lactic-lactate solution having Ph 3.52, 3.82 and 4.12 were prepared. Total concentration of lactic acid was

0.1 mol in all of these solutions. Titration values differed, however, as in 0.067, 0.05 and 0.33 mol. Solubility experiments with these solutions gave the following results.

TABLE V.

Solubility of enamel from persons 25 years old during 12 hours at 22°C, placed in a solution having a viscosity of 1.02.

No.	Titration Acidity	PH	$\frac{\text{Titration acidity}}{\text{PH}}$	Solubility (percentage)
1	0.067	3.52	19	27.04
2	0.05	3.82	13	18.00
3	0.033	4.12	8	12.41

We can perceive from the table that the solubility of enamel in lactic acid solution roughly varies as the ratio of $\frac{\text{total acidity}}{\text{pH}}$

6. *The factors influencing the solubility of enamel by fruit juices.*

In considering the results of the effect of PH value, viscosity and titration value of acid solution on the solubility of enamels, these values were measured in the fruit juices mentioned under section 2. The titration value was determined either with 0.1 or 0.02N NaOH solution.

TABLE VI.

Sort of Juice	PH	Titration value	Viscosity	$\frac{\text{Titration value}}{\text{PH} \times \text{viscosity}}$	Solubility percentage
Lemon	2.4	1.14 N.	1.32	0.36	77.03
Vinegar	3.1	0.72	1.08	0.215	27.89
Citrus sinense	2.6	0.276	1.24	0.0856	18.85
Apple	3.7	0.068	1.37	0.0134	5.08
Grape	3.3	0.043	1.58	0.0082	2.47
Pear	4.4	0.028	1.70	0.00374	1.72
Grape of Italian origin	3.5	0.012	1.82	0.0019	1.45

It can be seen from the table that the solubility runs fairly parallal with $\frac{\text{titration value}}{\text{PH} \times \text{viscosity}}$, while the PH value of the juice alone fails to explain entirely the difference in the degree of solubility.

7) The difference in solubility of deciduous, adolescent and senile enamel and the comparison of solubilities between enamels obtained from persons different in susceptibility against caries.

Pieces of enamel were obtained from persons aged 11 (deciduous second molar), 25 and 55, and their solubility in acidic solution was compared. The difference in solubility was also compared between enamels obtained from persons almost completely immune to caries and those who were quite susceptible to caries.

0.7 N acetic acid was used as an acid solution. The PH value was 2.5 at the beginning of the experiment and did not differ much after each experiment. The viscosity of the solution was 1.073. The duration of time was 12 hours. The result is shown in table 7.

TABLE VII,

Sort of enamel	weight of enamel		Solubility Percentage
	before	after	
Deciduous	107.63 mg	53.30 mg	50.47
Young	208.84 mg	139.78 mg	33.06
Old	160.32 mg	103.55 mg	35.41
Relatively immune	718.91 mg	493.46 mg	31.36
Susceptible	640.22 mg	412.49 mg	35.57

D. Summary.

1. The enamel is not affected by a solution at a PH value higher than 6.77, even after fifty days at 37°C.
2. The solubility of enamel is dependent upon
    a) hydrogen ion concentration,
    b) the titrable acidity in case of the same PH value, and
    c) the fluidity of the solution.
3. The deciduous enamel is most soluble in acid.

4. Among the permanent enamels the adolescent ones are less soluble in acid than the senile ones.

5. The solubility of the enamel obtained from persons highly susceptible to caries is greater than those from persons almost completely immune to caries.

II. Some Physico-chemical Characteristics of Saliva.

For many years it has been considered that various abnormal conditions of the teeth and the soft tissue of the oral cavity are associated with changes in salivary secretion. There is, however, a divergence of opinions. Some investigators state that saliva protects teeth from caries, while others ascribe the occurrence of caries to the deleterious effects of salivary composition.

To find a possible relation, if any, between the nature of saliva and caries susceptibility, I proceeded to examine some of the physico-chemical characteristics of saliva from about 2000 people, the majority consisting of school boys and girls, with reference to the different susceptibility of individuals.

The physico-chemical characteristics examined in this experiment were the volume, viscosity, Ph and buffering value of saliva.

A total of 1904 persons were examined, selected from kindergarten, primary school (904), high school (376, 283) and college.

A. The methods of examination.

1) Without any preliminary cleaning of the buccal cavity, the saliva was collected into a clean glass stoppered bottle numbered for each individual, generally at 10 a.m. In the collection of saliva the use of any activating agent was avoided.

2) The Ph value was examined immediately after the collection using La-Mott's apparatus.

3) The quantity of saliva which was collected during 30 minutes was measured. Regarding the degree of secretion, cases over 16. cc were designated as large, while cases under 9. cc, as

small, and cases between 10. and 15. cc, as medium in all of the following considerations.

4) The viscosity was determined by Ostwald's viscosimeter. The cases in which relative viscosity amounted to over 4.0 were rejected from our examinations.

5) The buffering action of saliva was examined by Michaelis method using paranitrophenol as an indicator.

6) The examination was performed as soon as possible after collection and when longer time was needed owing to the large number of specimens collected, I kept them in an ice-box. Almost all examinations were always finished, even in these cases, within four hours after collection.

7) Special attention was always paid to the character of the teeth of each individual, and the number of caries was noted, filled and crowned teeth being also counted as carious ones.

B. The result of examination.

Out of a total of 1904 persons 895 were males and 1009 females. A total of 1811 persons were affected with caries, of whom 833 were males and 978 females. The percentage of affection was therefore 95.1%, males 93.1%, females 96.9%.

The total number of teeth examined were 46234, 10844 of which being affected with caries, the percentage of affections, therefore, being 23.5%.

The quantity of saliva collected for 30. minutes averaged 14.8 cc. The relative viscosity varied between 1.05 and 3.12, averaging 1.31.

The PH value was between 6.10 and 7.73, with an average

TABLE VIII.

Sex	amount of saliva	viscosity of saliva	PH value of saliva	buffering power of saliva	percentage of caries	number of persons examined
Male	15.4	1.31	7.03	15.2	20.9	895
Female	14.2	1.31	6.75	13.8	25.5	1009

of 6.88. The buffering power varied considerably, changing between 5.0 and 25.6 with an average of 14.5.

The average values for both sexes are given in table VIII.

1. *The relation of the degree of carious affection with physico-chemical characteristics of saliva.*

The results of all the examinations were classified according to the degree of carious affection. People with less than 3 carious teeth were considered "relatively immune"; those with 4–7 affected teeth, "medium affected"; and those having more than 8 affected teeth, "highly affected".

As table IX indicates, the saliva of persons completely or almost completely immune to caries has a larger volume, greater

TABLE IX.

	Volume of saliva	Viscosity of saliva	PH of saliva	Buffering power	Number of persons examined
relatively immune	15.2 cc	1.33	7.06	15.7	658
medium affected	14.8 cc	1.31	6.80	14.2	716
highly affected	14.2 cc	1.28	6.70	13.3	530

TABLE X.

Factors		Percentage of carious teeth
Volume of saliva for 30 minutes	16 — 35 cc	22.5
	5 — 15 cc	24.5
Relative viscosity of saliva	more than 1.31	21.2
	less than 1.30	25.0
PH value of saliva	over 6.88	19.2
	below 6.87	28.4
Buffering power of saliva	higher than 15.0	19.2
	lower than 14.9	26.9

viscosity, higher PH value and stronger buffering power than that of persons inclined to caries affection.

When we re-classify the results according to the degree of volume, hydrogen ion concentration, viscosity and buffering action of saliva, we find again the same intimate relation among volume, viscosity, hydrogen ion concentration and buffering action of saliva and the degree of caries, as is shown in Table X.

2. *The relation of age and the degree of carious affection with reference to the change of physico-chemical characteristics of saliva.*

In table **11** and figure **1**. are shown the change of the above mentioned characteristics with the age of individuals.

As can be seen from the table and figure, girls in almost all ages secrete a smaller amount of saliva with a smaller PH value and a lower buffering power than boys of the same age. In viscosity alone there is no significant difference seen between both sexes. The percentage of carious teeth is also greater in girls than in boys, except at the ages of 8–12, when girls are a little less affected than boys. This exception at the ages of 8–12 is probably due to the earlier occurence of the exchange of deciduous teeth with permanent ones in girls. The minium of carious affection in both sexes coincides with the time of the completion of the exchange period.

C. Summary.

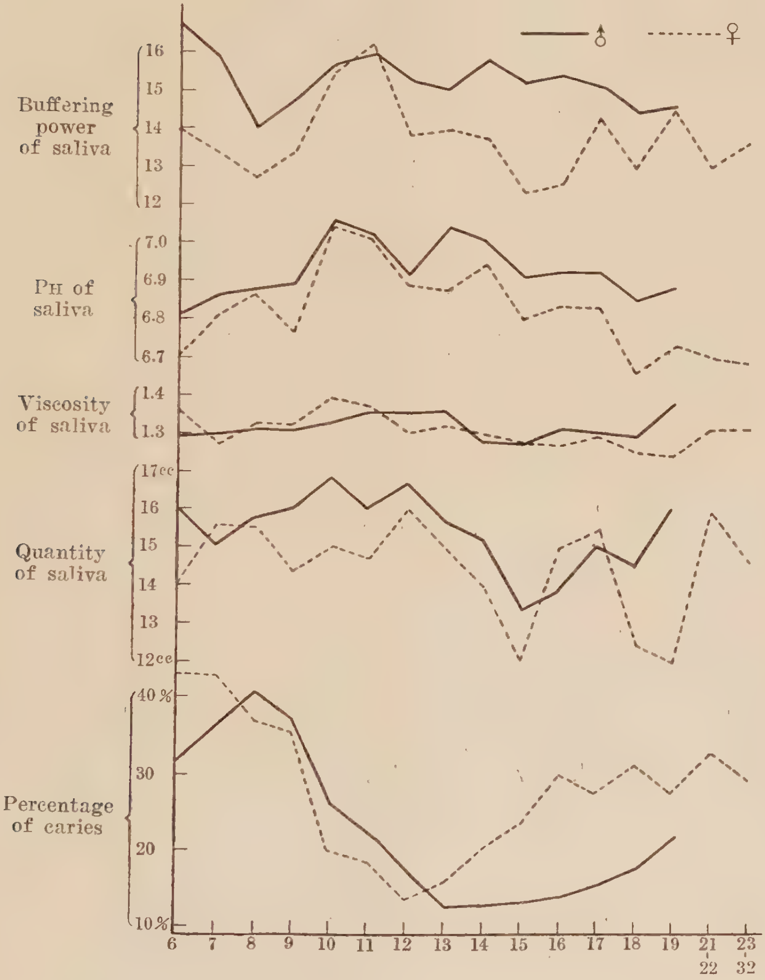
1. Persons secreting larger quantities of saliva of greater viscosity, higher PH value and stronger buffering power are less affected with caries than those secreting saliva lower in these qualities.

2. Girls are more affected in caries than boys of the same age, except at the exchange period.

TABLE XI.

Age	Sex	Number of People	Quantities	Viscosity	PH value	Buffering Power	Percentage of carious affection
6	Male	22	16.0 cc	1.29	6.91	16.7	30.8
	Female	14	14.0 cc	1.36	6.80	13.9	43.1
7	”	55	15.0 cc	1.29	6.96	15.9	35.6
	”	93	15.5 cc	1.27	6.91	13.4	43.0
8	”	111	15.7 cc	1.31	6.97	14.0	40.2
	”	80	15.5 cc	1.32	6.97	12.7	37.0
9	”	81	16.0 cc	1.31	6.96	14.8	37.0
	”	63	14.3 cc	1.32	6.87	13.4	35.7
10	”	42	16.8 cc	1.33	7.16	15.6	26.0
	”	51	15.0 cc	1.39	7.16	15.4	19.5
11	”	79	15.9 cc	1.36	7.12	16.0	22.7
	”	85	14.7 cc	1.37	7.12	16.2	18.4
12	”	46	16.6 cc	1.35	7.01	15.3	17.1
	”	35	16.0 cc	1.30	6.99	13.8	13.2
13	”	49	15.7 cc	1.36	7.14	15.1	12.0
	”	112	15.1 cc	1.32	6.98	14.0	15.3
14	”	72	15.2 cc	1.29	7.11	15.9	12.1
	”	186	14.1 cc	1.30	7.05	13.8	19.8
15	”	133	13.3 cc	1.28	7.02	15.3	12.4
	”	53	12.0 cc	1.28	6.91	12.4	23.0
16	”	96	13.9 cc	1.31	7.03	15.5	13.4
	”	31	15.0 cc	1.27	6.94	12.6	29.2
17	”	68	15.1 cc	1.31	7.03	15.3	14.9
	”	12	15.5 cc	1.30	6.94	14.4	27.0
18	”	25	14.5 cc	1.30	6.95	14.6	17.0
	”	39	12.5 cc	1.26	6.77	13.1	30.6
19	”	16	16.0 cc	1.38	6.99	14.7	21.0
	”	28	12.0 cc	1.25	6.84	14.7	27.0
21–22	Female	80	15.9 cc	1.32	6.81	13.1	32.2
23–32	”	47	14.7 cc	1.32	6.80	13.8	28.3

Fig. 2.



III. THE ACIDIC FERMENTATION IN ORAL CAVITIES AS A FACTOR IN THE DEVELOPMENT OF CARIES.

Under sections I and II I have examined the solubility of enamel in acidic solutions and the nature of saliva as the possible factors influencing the development of carious tooth. As the third factor I shall consider the degree of introduction or production of acidic substances in oral cavities. If we constantly eat strong acidic substances, it is quite possible that our teeth are apt to be corroded. The same dangerous effects may also occur when the acidic fermentation of sugar substances takes place constantly in an oral cavity.

As is already known many kinds of mouth microorganisms are capable of splitting sugar stuffs into lactic and other acids.

The kinds and amount of these microorganism vary a great deal with the constitutional and nutritional conditions of the individual and also with the degree of dental aid.

It seems quite natural to suppose that under the same condition of teeth and saliva, the development of caries may also be promoted by the degree of acidic fermentation.

In this experiment the powers of acidic fermentation of mouth washings, obtained under definite conditions from 1267 individuals, were examined and their results were compared with the degree of carious affection.

A. The collection of mouth washings.

Each individual was asked to held ca 12. cc of artificial saliva, prepared by the mixture of 78.2 milligram percent of K, 46.0 of Na, 12.0 of Ca, 1.2 of Mg, 161.9 of Cl, 13.6 of PO_4 and 1.9 of KCNS. having PH 6.0, and to clean the surface of the teeth five times with a dry sterilized brush. All washings were put into separate bottles.

The collected washings were always opaque, colorless or slightly colored, and had PH 6.0–6.2.

B. Examination of acidic fermentability of mouth washings.

3. cc of the collected mouth washing were added to 10. cc of artificial saliva, above mentioned, containing 5 percent glucose or cane sugar. The mixture was placed in an incubator at 37°C for 48 hours and 10. cc of the mixture was titrated with $\frac{N}{100}$ NaOH to determine the amount of acidic aubstance produced.

It may be mentioned that both the sugar solution and the artificial saliva were sterilized in Schimmelbush for 45 minutes.

C. The comparison of the percentage of carious teeth with the acid fermenting power of mouth washings.

In the following the persons were divided into three groups according to the number of carious teeth. People with less than 3 carious teeth were called "immune;" those with 4–7 affected teeth, "medium affected;" and those having more than 8 affected teeth, "highly affected"

In table XII, the PH values of fermentation mixture and the volume of NaOH used for neutralization of acid produced are shown according to the three grades of carious affections.

TABLE XII.

Degree of carious affection	Number of caries Percentage	Glucose		Cane Sugar	
		PH	NaOH used for neutralization	PH	NaOH used for neutralization
relatively immune	8.2 %	3.40	4.06 cc	3.41	4.18 cc
medium affected	21.8 %	3.25	4.68 cc	3.32	4.55 cc
highly affected	43.8 %	3.09	5.51 cc	3.20	4.62 cc

The table indicates clearly the fact that people whose mouth washings have higher fermentating power with a greater production of acid are apt to be highly affected with caries.

D. The comparison of the number of treated carious teeth in persons with artificially sound sets of teeth with the acid fermenting power of mouth washings.

In the preceding section it was shown that the more carious affected person produces more acid fermenting mouth washings. With the possibility in mind that the carious cavity may be a resort for microorganisms, the relation above mentioned may represent only the result of the caries cavity for the prodution of fermenting mouth washing and may not indicate the causual relationship of fermenting power to induce carious teeth. To exclude this possibility, cases were pick up, in which carious teeth were all treated, either filled or crowned, and a comparison made as in the previous section. The result is shown in table 13.

TABLE XIII.

Degree of carious affection	Number of artificially sound teeth	Glucose		Cane Sugar	
		PH	NaOH used for neutralization	PH	NaOH used for neutralization
relatively immune	6.3 %	3.39	4.32 cc	3.46	4.03 cc
medium affected	21.9 %	3.32	4.47 cc	3.42	4.33 cc
highly affected	36.3 %	3.12	5.32 cc	3.21	4.59 cc

From the table it can be said definitely that people who are apt to be affected with caries, produce more acid fermenting washings even when the whole set of teeth is completely treated.

E. The relations of PH and titration value with carious affection.

In the following tables, persons were divided into groups

TABLE XIV.

1). PH value and percentage of caries.

PH of fermented mixture	Number of persons examined		Percentage of caries	
2.8	Male	35	Male	40.3 %
	Female	32	Female	38.3 %
	Total	67	Average	39.3 %
3.3	Male	66	Male	22.6 %
	Female	78	Female	22.6 %
	Total	144	Average	22.6 %
3.8	Male	12	Male	17.3 %
	Female	17	Female	17.6 %
	Total	29	Average	17.5 %

2). Titrable acidity and percentage of caries.

Amount of N/100 NaOH used for neutralization taken in average in two groups with greater or less than 4.84	Number of persons examined	Percentage of caries
6.31	358	31.2
3.78	499	22.5

according either to PH value or to titrable acidity of the fermented glucose mixture given by mouth washings, and the percentage of carious teeth in those groups was recorded together.

From this table it can be said that persons with mouth washings capable of more acid producing power suffer more from dental caries.

F. Acid producing power of mouth washings in different ages of both sexes.

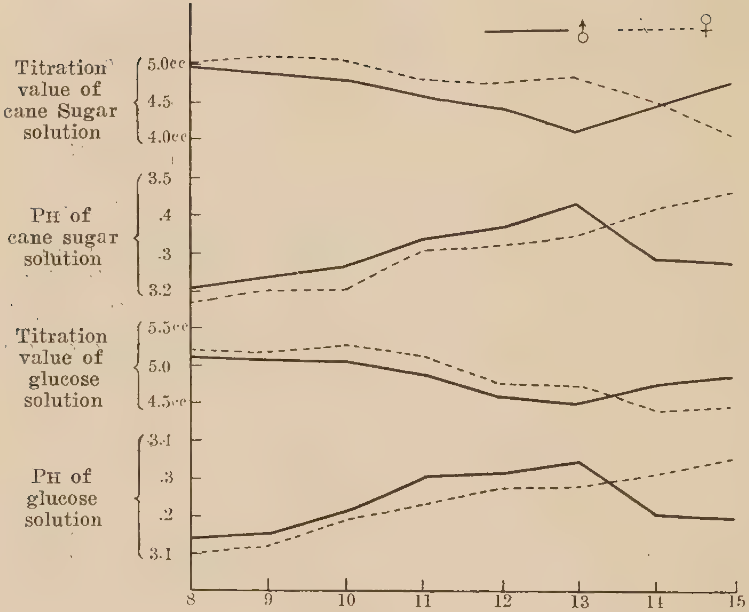
In the following table and figure are recorded the average of acid producing powers of mouth washings in different ages in both sexes.

Table XV.

Ages	Number of persons examind		Amount of N/100 NaOH used for titration			
			in fermented glucose solutions		in fermented cane sugar solutions	
	Male	Female	Male	Female	Male	Female
8	56	51	5.14	5.19	4.96	5.01
9	68	62	5.11	5.17	4.83	5.07
10	51	50	5.04	5.24	4.75	5.05
11	50	51	4.84	5.10	4.53	4.81
12	51	58	4.53	4.75	4.36	4.70
13	52	52	4.45	4.69	4.01	4.74
14	51	50	4.72	4.35	4.39	4.45
15	50	54	4.80	4.38	4.60	3.96

As can be seen from the table and figure, the acid fermenting power of girls always predominates that of boys.

Fig. 3.



G. Summary.

1. The mouth washings of the person suffering little from dental caries have small acid producing power when digested with sugar solution and vice versa.

2. The acid producing power of mouth washings is stronger in girls than in boys.

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EFFECTIVENESS OF STROMA PROTEIN ON THE PRODUCTION OF HEMOLYSIN.

By

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I. Introduction.

Since the fact was confirmed by Bordet (1900), that the hemolysinogen of red blood corpuscles exists in the stroma and not in the hemolyzed liquid, attempts were made by many investigators to find the effective constituent of stroma that could induce the hemolysin formation in the organism injected with it. Attempts were made to extract the effective substance with ether or boiling benzene from ox erythrocytes or stroma by Bang and Forssman (1906) and Takaki (1908), and its near relation to lipoid was determined by them from its solubility. Landsteiner and v. d. Sheer (1925) found the hemolysin inducing power of the mixture of alcoholic extract of horse erythrocytes and pig serum, when injected into rabbits, while each of these two components separately had no effectiveness. They considered that the alcoholic extract was lipoid and this behaved as hapten when mixed with pig serum as a schlepper. The same results were obtained by Misawa (1925), Sakamoto (1928) and Tobaya (1929) with the erythrocytes of many animals and they concluded that the erythrocytes of some animals had only lipoid hemolysinogen, some only protein and some both lipoid and protein.

The stroma of erythrocyte is composed of lipins and proteins intimately bound and the mere treatment with alcohol, hot benzene, etc., is hardly sufficient to separate these two components completely. It is, therefore, still a question to decide whether we are able to anounce the chemical nature of the lysinogenic power so far as the above researches are concerned. I have tried, therefore, under the guidance of Professor S. Kakiuchi, to study the effective hemolysinogenic constituent of stroma. For this purpose it is necessary to separate the protein and the lipin of stroma as completely as possible under a mild treatment. As was formerly shown by Kawai (1929) of this Institute, the hemolysis induced by means of the photosensibilization with hematoporphyrin is probably due to the dissolution of the colloid-chemical association between lipin and protein. I have, therefore, tried to separate the lipin and the protein fraction by this photosensibilization method and examined the hemolysinogenic character of each fraction. The results of the experiments are given in the following sections.

II. IMMUNIZATION EXPERIMENT WITH OX ERYTHROCYTE STROMA.

A. Preparation of stroma.

600 cc. of defibrinated ox blood, filtered through cotton-wool, was well mixed with the same volume of 0.85 % sodium chloride solution. Erythrocytes were then separated as much as possible from the mother liquor by centrifugation, and washed three times with the addition of 900 cc. of 0.85 % sodium chloride solution and subjected to centrifugation each time. The erythrocytes obtained were hemolyzed with 2400 cc. of water, and the stroma was precipitated from the solution by the addition of 2000 cc. of 0.01 n hydrochloric acid. The precipitate was repeatedly washed with water, until no colouring substance exists in the supernatant liquid. Then the mixture was neutralized with 0.01 N sodium hydroxide solution, when a uniform suspension of stroma was obtained. To this solution 0.01 n hydrochloric acid was added in order to precipitate the stroma again, and the same procedure of washing was repeated as above, until the stroma precipitate became entirely colourless. Neutralized with 0.01 n sodium carbonate solution, 220 cc. of 1 % stroma suspension were obtained. Evaporated in vacuum it gave an almost

colourless powder, which contained 11.18 % of nitrogen (determined by micro-Kjeldahl). It is precipitated from its neutral or alkaline medium completely at pH 5.6 on the addition of acid.

B. Method of immunization.

5 cc. of the above obtained stroma solution were injected intravenously into rabbits weighing 2.5–3 kg at intervals of 6—7 days after 3 and 6 injections the hemolysin titers of the inactivated sera were tested, by adding 0.2–0.5 cc. of 10 fold diluted guinea-pig serum, corresponding to 2 units of complement, and 0.5 cc. of 2.5 % suspension of washed ox blood corpuscles to 0.5 cc. of progressively diluted serum. The normal hemolysin against ox blood present in the guinea-pig serum was removed previously by absorption with ox blood corpuscles at low temperature. After the lapse of 2 hours in an incubator at 37°C., the degree of hemolysis was examined. The agglutinin titers were also determined by adding one drop of 2.5 % suspension of washed ox blood corpuscles to each 0.5 cc. of progressive dilutions of the inactivated immune sera and examining the agglutination of the erythrocytes after 2 hours in the incubator.

In the following tables the agglutinin or hemolysin titer is given by the highest dilution of tested serum in which agglutination or complete hemolysis occured.

C. Results.

The effectiveness of stroma on the production of hemolysin and agglutinin was proved as shown in Table I.

TABLE I.

Antibody inducer	No. of Rabbit	Hemolysin titer			Agglutinin titer		
		before injection	after 3 injections	after 6 injections	before injection	after 3 injections	after 6 injections
Stroma	1	0	1600	400	5	400	
	2	0	800	400	1	100	200
	3	0	800	800	0	100	400

III. SEPARATION OF LIPIN AND PROTEIN OF STROMA.

A. Irradiation of stroma in the presence of hematoporphyrin.

Hematoporphyrin was added in the concentration of 0.004 % into 200 cc. of the 1 % suspension of stroma. After the lapse of 1.5 hours in the dark place the mixture was irradiated within a Petri's dish with 1000-Watt and 1500-Watt electric lamps at a distance of 50 cm., avoiding heat effects by cooling with an electric fan. The effectiveness of hemolysin inducing power did not diminish when the duration of irradiation remained one hour, as is shown in the following table.

TABLE II.

Antibody inducer	No. of Rabbit	Hemolysin titer			Agglutinin titer		
		before inj.	after 3 injs.	after 6 injs.	before inj.	after 3 injs.	after 6 injs.
10 minutes irradiated stroma	4	0	100	100	0	100	400
	5	0	400	200	0	50	
	6	0	50	100	0	20	
30 minutes irradiated stroma	7	0	100	200	5	5	
	8	0	800	200	2	100	
	9	0	200	200	2	20	
1 hour irradiated stroma	10	0	400	100	2	20	
	11	0	50	200	2	100	
	12	0	200	200	2		
3 hours irradiated stroma	13	0	100	20	5		
	14	0	800	200	1		
	15	0	20	20	1		

B. Extraction of the lipin fraction of stroma.

The irradiated stroma suspension was made pH 3 by the adequate addition of 0.1 n hydrochloric acid and mixed well with the same volume of alcohol. The mixture was then added to the same volume of ether and petroleum ether and well shaken.

The ether-petroleum ether portion was removed, and the water portion was repeatedly treated as above with ether and petroleum ether, until nothing is extracted in the ether portion at all. The water portion was concentrated, neutralized with 0.1 n sodium carbonate, and the dilution was adjusted to get a 1 % solution of stroma protein.

The ether-petroleum ether portions were brought together and evaporated to dryness in vacuo at 40°C. in a slow current of carbon dioxide. The dried extract was dissolved in a mixture of 1 volume of alcohol, 3 volumes of ether and 3 volumes of petroleum ether, filtered and the solvent was evaporated. The chloroform solution of this dried residue was extracted repeatedly with water at the alkaline reaction with the addition of alcohol in order to remove the mixed hematoporphyrin, etc. Chloroform was evaporated, and the dried residue was dissolved in water-free ether. The insoluble portion was removed by centrifugation, and the solvent was evaporated. The residue was again dissolved in pure petroleum ether, centrifuged and the supernatant liquid was evaporated. By this treatment from 6800 cc. of ox defibrinated blood 7.78 gm. of pure lipin was obtained.

IV. THE IMMUNIZATION EXPERIMENT WITH THE LIPIN FRACTION OF STROMA.

A. Chemical properties.

The obtained lipin portion is readily soluble in ether, petroleum ether or chloroform, and precipitates partially from its solution by the addition of acetone or alcohol. It is soluble in hot alcohol. Both Salkowski's reaction and Liebermann-Burchard's reaction are positive. Molisch's reaction and orcinol reaction are negative. The content of nitrogen is 1.16 %, determined by micro-Kjeldahl method, and phosphorous, 2.23 % by Fiske-Subbarow's method. Thus the ratio of P:N is 1 : 1. 15.

B. Method of Immunization.

2 cc. of 10 % alcoholic solution of lipin fraction were poured

quickly into 20 cc. of ten fold diluted pig serum. Sodium oleate Merck was added in amount of 0.5 % to secure the emulsification of lipin. After a lapse of 1–2 hours at room temperature, 5 cc. of it were injected into the marginal vein of a rabbit's ear. As a method of control the lipin solution without pig serum or pig serum without lipin fraction was prepared and injected in the same manner. The injections were carried out every 5–7 days and on the 5th–7th day after 3,6 and 9 injections the blood was taken out and examined for hemolysin and agglutinin.

C. Results.

The results obtained by the immunization with the lipin fraction of stroma are as follows.

TABLE III.

Antibody inducer	No. of Rabbit	Hemolysin titer				Agglutinin titer			
		before inj.	after 3 injs.	after 6 injs.	after 9 injs.	before inj.	after 3 injs.	after 6 injs.	after 9 injs.
Lipin fraction and pig serum (sodium oleate added)	16	0	0			0	0		
	17	1	0	0		0	0	0	
	18	0	0	0	0	0	1	2	2
	19	0	0	0	0	0	0	1	2
	20	0	0	0	0	0	0	0	0
Lipin fraction (sodium oleate added)	21	0	0			0	0		
	22	0	0	0		0	0	1	
	23	0	0	0	0	0	0	1	1
Pig serum (sodium oleate added)	24	0	0	0	0	0	0	1	0
	25	0	0	0	0	1	2	1	1
Pig serum alone	26	0	0	0	0	1	1	1	2
	27	0	0	0	0	1	2	0	0

From Table III we can see that the lipin fraction of stroma has no lysinogenic or agglutinogenic power, not only when injected alone, but even when injected with pig serum as a schlepper.

V. EXPERIMENT WITH THE PROTEIN FRACTION OF STROMA.

A. Chemical properties.

The water portion obtained by the method described under experiment III is a colloidal solution, and is precipitated within the range of pH 6-5 and dissolved by the addition of alkaline. Precipitation tests and colour tests for protein are both positive. It contains 15.45 % of nitrogen. The amount of lipin which was estimated by the Bloor's oxydimetric method applied after the saponification by the Kumágawa-Suto's method, was 0.021 %.

B. Immunization experiment with the protein fraction.

5 cc. of 1 % solution of protein fraction containing 0.85 % sodium chloride were injected intravenously. In some experiments the influence of the addition of 1/10 volume of pig serum to the protein fraction was also investigated. The results obtained are shown in Table IV.

TABLE IV.

Antibody inducer	No. of rabbit	Hemolysin titer		Agglutinin titer	
		before injection	after 3 injections	before injection	after 3 injections
Protein fraction	28	0	512	0	16
	29	0	256	2	32
Protein fraction and pig serum	30	0	64	2	16
	31	0	512	0	256
	32	0	64	0	32

Table IV proves that hemolysinogen and agglutinogen exist in the protein fraction and the addition of pig serum seems to have no influence on the effectiveness of these antigens.

C. Immunization experiment with the mixture of lipin and protein fractions.

0.2 gm. of lipin fraction was dissolved in warm alcohol and poured quickly into 20 cc. of 1 % solution of protein fraction. Sodium oleate was added in amount of 0.5 % to secure the emulsification of lipin. After a lapse of 2 hours at room temperature 5 cc. of it were used for intravenous injection. The injection was repeated every 5–7 days and on the 5th–7th day after 3 and 5 injections the hemolysin and agglutinin titers were estimated. The results are shown in the following table.

TABLE V.

Antibody inducer	No. of Rabbit	Hemolysin titer			Agglutinin titer		
		before inj.	after 3 injs.	after 5 injs.	before inj.	after 3 injs.	after 5 injs.
Lipin fraction and protein fraction (sodium oleate added)	33	0	32	16	0	4	16
	34	0	16		1	64	
	35	0	2		1	16	
	36	0	32		1	16	
	37	0	16		0	0	
Protein fraction (sodium oleate added)	38	0	32	64	2	16	128
	39	0	128	256	1	2	32
	40	0	64	16	1	8	32
	41	0	64		1	1	

From this result it is observed that the mixture of the protein and lipin fractions gives no stronger lysinogenic and agglutinogenic effectiveness than the protein fraction alone.

CONCLUSION.

1. The lipin fraction of ox erythrocyte stroma shows no action as hemolysinogen and agglutinogen, even when pig serum is added as a schlepper.

2. The protein fraction of ox erythrocyte stroma, from which lipin is removed as completely as possible, acts still as a strong hemolysinogen and agglutinogen.

3. The hemolysin- and agglutinin-inducing action of ox erythrocyte stroma depends on the antigenic property of the protein portion, whereas the lipin portion seems to have no

influence on it.

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STUDIES ON THE MECHANISM OF CREATINE FORMATION. I.

By

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I. INTRODUCTION.

The mechanism of creatine formation within the animal body, is still quite unknown, notwithstanding the effort of many previous investigators. We have hitherto almost no idea what substance is its precursor and at what organ or tissue it is formed. I have therefore made some inquiry into these questions. The first difficulty I met was the estimation of creatine in blood, organs etc. Since Folin in 1904 reported his colorimetric determination of creatine, various modifications of this method were attempted by many investigators, without however reaching to any perfect method. In the following I am going to report my modified method of creatine determination, which I believe to be more satisfactory than all of the former methods, though I have not examined the new 3,5-dinitrobenzoic acid method recently reported by Benedict and Behre (1936). Some questions on the formation of creatine during autolysis, survivance and perfusion of rabbit liver with and without the addition of different substances were studied by my method and results so far as obtained are also given.

II. METHOD OF DETERMINATION.

To estimate the total creatinine in urine, blood or tissues, the methods of Folin (1914), Myers and Fine (1915), Janney and Blatherwick (1915), Hammet (1921), Rose, Helmer and Chanutin (1927) etc. are used at present. In my method, however, creatine-body was extracted with trichloracetic acid at room temperature avoiding the treatment of extraction by heat in acidic solution to reduce the production of chromogenic substances to the minimum. Furthermore, the filtrate was treated with lead acetate, which removed the chromogenic substances leaving creatine-body in solution. In some cases the total creatinine can be estimated roughly from trichloracetic acid filtrate without treatment with lead acetate, though the lead-treatment gave always a lower value than without it (see Table I and II). The difference was considerably great in liver, while it was very small in muscle. That the treatment with lead acetate gives no loss in creatinine estimation was proven by the determination of creatine or creatinine added to the original test material. That the creatine-body can be extracted with trichloracetic acid almost completely is obvious from the fact that there was no great difference in the amount of total creatinine between the one which was previously heated with acetic acid in an autoclave at 120°C. for 45 minutes (by this treatment creatine, at least for the most part, is transformed to creatinine) and then treated with lead acetate, and the other in which the material was extracted with trichloracetic acid and the extract treated with lead acetate, followed lastly by heating in an autoclave at 120°C. for 45 minutes. The values of the total creatinine of both cases were respectively 15.4 mg % and 14.9 mg % in liver.

1. *Extraction of creatine-body.*

A definite amount of tissue (about 3 gm. in case liver is used for determination) is rubbed with about 2 gm. of purified sea sand and a small quantity of water to a fairly uniform paste in a mortar, 5 cc. of trichloracetic acid solution (566 gm. per liter) and about 15 cc. of water are gradually added to the paste, while the rubbing and stirring is continued for ten minutes. By this treatment the proteins are converted into insoluble precipitates and the creatine-body goes in solution. The mixture is then transferred to a 50 cc. volumetric flask by the aid of some amount of water and the flask is filled up to the mark with water. Thoroughly mixed, after standing for one hour or longer, the mixture is filtered. This filtrate is better treated with lead acetate as described below. However, the total creatinine can be roughly estimated without treatment as follows:

15 cc. or 20 cc. of the filtrate are transferred into a 30 cc. flask with short neck and glass-stopper and 10 % NaOH is added to it to bring its pH to about 9.4. The mixture is filled up to the mark with water and thoroughly shaken and filtered. 10 cc. of this filtrate are treated with 2N HCl in a glass cylinder graduated at 10 cc. The succeeding procedures are performed as described below.

2. *Lead acetate treatment and estimation of total creatinine.*

15 cc. or 20 cc. of the above mentioned filtrate are measured into a 30 cc. glass-stoppered and short-necked flask and 2 cc. of lead acetate solution (crystalline lead acetate 35 gm. dissolved in 100 cc.) are added to it. Then 10 % NaOH is gradually added under shaking to the mixture to make its pH to about 9.8. The lead is then almost completely precipitated, carrying with it chromogenic substances other than creatine-body. The mixture is filled with water to the mark, mixed well and after standing for 15 minutes filtered. Though a small amount of lead in the filtrate, effects no hindrance for colorimetric determination, larger quantities must be removed by the addition of a drop of phosphate solution (KH_2PO_4 10 gm./50 cc.). 10 cc. of clear and colourless filtrate thus obtained are treated with 2N HCl in a 10 cc. glass cylinder (1 cc. of 2N HCl is to be added for every 10 cc. of the filtrate, and besides, a certain excess of 2N HCl equivalent to trichloracetate and acetate dissolved in it must be added). The cylinder is then covered with a glass lid and heated in an autoclave at 120°C. for 45 minutes. When cooled below 100°C. the autoclave is opened, and the contents of the cylinder are evaporated on a water-bath to about 8 cc. The mixture is neutralized with 10 % NaOH and filled with water to the mark.

10 cc. of the solution thus obtained are treated with 5 cc. of a saturated solution of purified picric acid and 1 cc of 10 % Na OH. At the same time 10 cc. of a standard creatinine solution are treated with picric acid and alkaline solution just the same as the test solution. After standing for 8 minutes the colour comparison is made as usual with the standard at 20 mm.

The value of the total creatinine thus obtained may not always result from creatine-body alone. At least, it is impossible to remove glycocyamine by this lead-treatment from the solution containing it. The values in all experimental results are therefore called as total creatinine-equivalent instead of total creatinine.

III. THE CREATINE-BODY CONTENT OF RABBIT LIVER IN THE CASE OF AUTOLYSIS AND SURVIVANCE.

It was reported by Myers and Fine (1915) that the total creatinine increased during 10 days autolysis of liver without any addition, and the same increase was shown by Abderhalden and Möller (1927) during 36–48 hours autolysis of paste of liver and muscle under the addition of choline and arginine. In this experiment I have examined the question whether or not the creatine-body really increases during the autolysis of liver paste.

1. *The effect of the autolysis of long duration of rabbit liver paste on the total creatinine-equivalent.*

The rabbit was bled from the carotid artery and the liver was taken out as promptly as possible without washing. The liver was freed from connective tissue and ground in a mortar to a moderately uniform paste without sand after wiping up the blood adherent to it with dry clean cloth. About 3 gm. of this paste were weighed into a glass-stoppered cylinder and 15 cc. either of physiological saline solution, or modified Ringer's solution (carbonate-free Ringer's solution 100 cc. + 13 gm./l. $NaHCO_3$ solution 4 cc.) or m/15 phosphate buffer solution (pH

7.38) were added to it. The cylinders were then allowed to stand in an incubator at 37°C. for a certain period under the addition of toluene to prevent contamination and the total creatine-equivalent was estimated every three days. The total creatinine-equivalent was estimated as mentioned above with a slight modification by extracting the residual part of autolysate first as usual within a mortar and then the fluid part added to it after. The result of the experiment shown in table I is a sample of autolysis which was conducted in the phosphate buffer solution.

TABLE. I

The change of the total creatinine-equivalent during autolysis of rabbit liver paste for a long period.

Period of autolysis (days)	Total creatinine-equivalent in liver (mg. %)			
	A		B	
	Rabbit 1	Rabbit 2	Rabbit 1	Rabbit 2
0	19.7	17.9	14.9	12.6
3	21.2	21.5	15.7	14.1
6	23.1	26.2	16.6	15.8
9	24.5	30.1	17.3	17.4

A: Values untreated with lead acetate.
B: Values treated with lead acetate.

As can be seen from the table the total creatinine-equivalent increases at a very uniform rate in the course of time, but the degree and rate of increase varies considerably case by case. In some cases a maximum of chromogenic formation was reached already before the 9th day. And if the contents of the cylinder were stirred by opening the glass-stopper, the total creatinine-equivalent was generally apt to increase rapidly. Such increase was especially great when the total creatinine-equivalent was determined without lead acetate treatment. Myers and Fine also recognized such an increase occurring in the course of the autolysis of rabbit liver. This increase of the total creatinine-equivalent with the lapse of time makes it very doubtful to decide whether the increase is really due to the creatine-body formation which is quite possibly produced by living tissue or rather due to the destructive process of autolysing enzymes. Therefore, I tried to study the experiment of short duration with sliced liver in which period liver cells seemed to maintain their vitality.

2. *The effect of the autolysis of liver paste of previously starved rabbit on the total creatinine-equivalent under a slow current of oxygen with constant shaking.*

To minimize the amount of chromogenic substance, I have first studied the effect of the starvation of a rabbit on the total creatinine-equivalent of its liver during autolysis. After rabbits were starved respectively for 0, 1, 2, 3, 4, 6 and 10 days, each liver was immediately extirpated and its paste was prepared as mentioned above. About 3 gm. of the paste were weighed into

TABLE II.

The change of the total creatinine-equivalent of starved rabbit liver paste during autolysis under oxygen supply with constant shaking.
(T. C. E. = Total creatinine-equivalent)

Rabbit No.	Period of starvation (days)	Before autolysis		After 6 hours autolysis			
		T. C. E. in liver (mg. %)		T. C. E. in liver (mg. %)		Increased T. C. E. in liver (mg. %)	
		A	B	A	B	A	B
3	0	15.0	10.6	17.4	11.0	2.4	0.4
4	1	15.1	9.6	15.9	9.8	0.8	0.2
5	2	21.7	15.5	22.9	15.8	1.2	0.3
6	3	11.6	7.4	13.5	7.6	1.9	0.2
7	4	19.7	13.3	21.3	13.7	1.6	0.4
8	6	29.4	22.7	33.4	23.6	4.0	0.9
9	10	31.6	24.5	35.3	25.4	3.7	0.9

A: Values untreated with lead acetate.
B: Values treated with lead acetate.

an aerating flask and then 15 cc. of modified Ringer's solution (carbonate-free Ringer's solution 100 cc. + 13 gm./l. $NaHCO_3$ solution 4 cc.) and 5 drops of toluene were added to it. The flask was shaken constantly in a thermostat at 38°C. by means of a shaking machine under a slow current of oxygen. After 6 hours the autolysate was ground with sand and treated with trichloracetic acid solution in a mortar. The total creatinine-equivalent of these liver pastes before and after autolysis was determined as described above. The results are shown in Table II.

As is clear from the table, even as short a duration of autolysis as 6 hours, the chromogenic substance (perhaps it will not be creatine-body) increases under such a condition. The least value of the total creatinine-equivalent is, however, found after 1, 2 or 3 days of starvation. I have, therefore, always used rabbits for further experiments after 2 days of starvation.

3. The effect of the incubation of sliced rabbit liver on the total creatinine-equivalent in case of the addition of various substances under a slow current of oxygen with constant shaking

As it seemed to me quite doubtful that the increased total creatinine-equivalent of the paste during autolysis was due to the production of creatine-body, and that it rather suggested the production of some chromogenic substance other than creatine-body, I have proceeded to perform the experiment with sliced rabbit liver for a short period. At first the oxygen consumption of sliced rabbit liver was estimated by Warburg's method to see how long the liver cells maintain their vitality. It was found that the sliced liver consumed oxygen distinctly for 3 hours under the experimental condition. Therefore, the period of experiment was limited to 3 hours in this kind of experiment. As it was found, on the other hand, that the toluene hindered the oxygen consumption of cells, the addition of toluene was omitted in all experiments for as short a period as 3 hours except in one case.

The fresh unwashed liver of a rabbit was sliced with a safty razor about 0.5 mm. thick. About 3 gm. of this sliced liver were weighed into an aerating flask and then 15 cc. of modified

TABLE IIIa.

The composition of the surviving mediums used for the experiments described on Table IIIb.

Symbol	Compositiou
A_1	M. R. $S._1$, 15 cc. + isot. $NaHCO_3$ 1 cc. + toluene, one drop.
A_2	„ + thyr. (0.05 mg.) in 1 cc. + toluene, one drop.
A_3	„ + thyr. (0.01 mg.) in 0.2 cc. + toluene, one drop.
A_4	„ + thyr. (0.005 mg.) in 0.1 cc.
A_5	M. R. $S._1$, 15 cc.
A_6	„ + glyc. (30 mg.) in 1 cc.
A_7	„ + glyc. (30 mg.) in 1 cc. + isot. $NaHCO_3$, 1 cc.
A_8	„ + glyc. (30 mg.) in 1 cc. + thyr. (0.05 mg.) in 1 cc.
A_9	„ + arg. (40 mg.) in 2 cc.
A_{10}	„ + isot. $NaHCO_3$ 2 cc.
A_{11}	„ + arg. (40 mg.) in 2 cc. + thyr. (0.05 mg.) in 1 cc.
A_{12}	„ + d-alanine (40 mg.) in 2 cc.
A_{13}	„ + adrenaline 0.5 cc.
A_{14}	„ + adrenaline 0.1 cc.
A_{15}	„ + adrenaline 0.5 cc. + thyr. (0.025 mg.) in 0.5 cc.
A_{16}	„ + adrenaline 0.1 cc. + thyr. (0.005 mg.) in 0.1 cc.
A_{17}	M. R. $S._2$ 15 cc.
A_{18}	„ + thyr. (0.075 mg.) in 0.5 cc. + arg.-glyc. 1 cc.

M. R. $S._1$ = Modified Ringer's $solution_1$ (composition: carbonate-free Ringer's solution 100 cc. + 13 gm./1. $NaHCO_3$ solution 4 cc.).

Isot. $NaHCO_3$ = Isotonic $NaHCO_3$ solution.

Thyr. = Thyroxine (E. R. Squibb & Sons (New York)): suspended in isot. $NaHCO_3$.

Glyc. = Glycocoll: dissolved in water.

Arg. = Arginine carbonate (prepared by the method of Arnold E. Pratt (1926) and purified): dissolved in water.

Adrenaline = Sankyo's adrenaline hydrochloride (1:1000) solution.

M. R. $S._2$ = Modified Ringer's $solution_2$ (composition: carbonate-free Ringer's solution 100 cc. + 13 gm./1. $NaHCO_3$ solution 3 cc.).

Arg.-glyc. = Arginine-glycocoll mixed solution (arginine carbonate 26 mg. and glycocoll 25 mg. were dissolved in 5 cc. of water).

TABLE IIIb.

The effect of the incubation of sliced rabbit liver on the total creatinie-equivalent in the case of the addition of various substances under a slow current of oxygen with constant shaking.

(T. C. E. = Tolal creatinine-equivalent).

Exp. No.	Rabbit No.	Symbol of the surviving medium. (See Tab. IIIb)	Main added substances to the surviving medium. (See Tab. IIIb)	Before incubation		After 3 hours incubation			
				Liver gm.	T.E.C. in liver. mg. %	Liver gm.	T.C.E. in liber. mg. %	Increased T.C.E. in liver. (mg. %)	Increased or decreased T.C.E. in liver by the added substances. (mg. %)
I	10	A_1	—	3.074	11.1	3.021	11.7	+0.6	
		A_2	Thyroxine	3.023	11.3	3.035	11.9	+0.6	+0.2
		A_3	Thyroxine			3.023	12.0		+0.3
	11	A_1	—	3.003	21.2	3.044	21.7	+0.5	
		A_2	Thyroxine	3.027	21.3	3.024	22.1	+0.8	+0.4
		A_4	Thyroxine			3.007	22.3		+0.6
II	12	A_5	—	3.005[1]	18.0	3,013[1]	19.4	+1.4	
		A_6	Glycocoll	3.073[1]	18.3	3.035[1]	19.5	+1.2	+0.1
III	13	A_7	Glycocoll	3.013	8.7	3.037	9.2	+0.5	
		A_8	Thyroxine, glycocoll	3.035	8.7	3.001	9.3	+0.6	+0.1
IV	14	A_5	—	3.027[1]	19.7	3.001[1]	20.4	+0.7	
		A_9	Arginine	3.003[1]	19.8	3.000[1]	20.4	+0.6	0
V	15	A_{10}	—			3.013	16.9		
		A_9	Arginine	3.011	16.3	3.030	16.7	+0.4	−0.2
		A_{11}	Thyroxine, arginine	3.033	16.2	3.009	16.9	+0.7	0
VI	16	A_5	—	3.029	16.3	3.039	16.9	+0.6	
		A_{12}	Alanine	3.028	16.3	3.073	17.1	+0.8	+0.2
VII	17	A_5	—	3.017	20.1	3.020	21.1	+1.0	
		A_{13}	Adrenaline	3.051	20.7	3.047	21.2	+0.5	+0.1
		A_{14}	Adrenaline			3.048	20.8		−0.3
VIII	18	A_5	—	3.042	10.2	3.054	10.4	+0.2	
		A_{15}	Adrenaline, thyroxine	3.008	10.2	3.039	10.5	+0.3	+0.1
		A_{16}	Adrenaline, thyroxine			3.011	10.6		+0.2
IX[2]	19		—	2.606	14.6	2.739	14.9	+0.3	
		A_{17} A_{18}	Throxine, arginine, glycocoll	2.541	14.6	2.504	14.9	+0.3	0

1) The liver was extirpated 70 minutes after the injection of thyroxine (1.3 mg./25 cc. isotonic $NaHCO_3$) suspension 2 cc. through the portal vein.

2) This experiment was carried out after the experiments of perfusion

Ringer's solution or equal volume of modified Ringer's solution together with or without the addition of a certain amount of testing substances were added to it (see Table III$_a$). The flask was constantly shaken in a thermostat at 38°C. by means of a shaking machine under a slow current of oxygen. After the lapse of 3 hours, the total creatinine-equivalent of the mixture was determined. The results are shown in Table III$_b$.

From Table III$_b$ it can be said that, though the total creatinine-equivalent after the incubation always increases, the increase caused by the presence of an added substance is relatively small, or is not found except in the case of glycocyamine. When glycocyamine is added, the increase of the total creatinine-equi-

TABLE IV.

The effect of the incubation of sliced rabbit liver on the total creatinine-equivalent in the case of the addition of thyroxine and glycocyamine under a slow current of oxygen with constant shaking.

Symbol of the surviving medium, (See below)	Before incubation			After 3 hours incubation				
	Liver gm.	T.C.E. mg.	T.C.E. of glycocyamine. mg.	Liver mg.	T.C.E mg.	T.C.E. of glycocyamine. mg.	Increased T.C.E. of glycocyamine mg.	Increased T.C.E. of glycocyamine per 100 gm. of liver. mg. %
A_{17}	2.548	0.422		2.515	0.435			
				(2.527)	(0.437)			
	(2.517)	(0.417)		(2.542)	(0.439)			
A_{19}				2.527	0.749	0.312	0.155	6.13
A_{20}	2.517	0.574	0.157	2.542	0.753	0.314	0.157	6.17

A_{17}: M. R. S.$_2$ = modified Ringer's solution$_2$ (composition: carbonate-free Ringer's solution 100 cc. + 13 gm./1. NaHCO 3 cc.) 15 cc.

A_{19}: M. R. S.$_2$ 15 cc. + glycocyamine (2 mg. in 1 cc. of 0.9% NaCl) solution 1 cc. (glycocyamine was prepared by the method of Nencki and Sieber (1878) and purified).

A_{20}: M. R. S.$_2$ 15 cc. + glycocyamine (2 mg. in 1 cc. of 0.9% NaCl) solution 1 cc. + thyroxine (0.15 mg. in 1 cc. of isotonic $NaHCO_3$) suspension 0.5 cc.

The figures in the brackets are calculated ones.

valent is considerably greater (see Table IV). On the other hand, when a definite amount of glycocyamine was allowed to stand with the sliced liver in a thermostat at 38°C. for 6 hours under aeration, and the glycocyamine-equivalent in the trichloracetic acid extract of the incubated material was determined by Weber's method (the modified Sakaguchi's method (1930)), there was definitely found a decrease of the glycocyamine-equivalent (see Table V). (This experiment was carried out before the period of incubation in all of our incubation experiments was decided for 3 hours.) To what substance does glycocyamine change during incubation ? Pherhaps glycocyamine is transformed to glycocyamidine, creatine-body or some other compound. However, I will dicuss this point in chapter IV and V, taking the result observed in the perfusing experiment into consideration.

TABLE V.

The effect of the incubation of sliced rabbit liver on glycocyamine added to the surviving medium.

Exp. No.	Before incubation			After 6 hours incubation		
	Liver gm.	Glycocyamine-equiv. gm.		Liver gm.	Glycocyamine-equiv. mg.	
		Found	Calculated		Found	Calculated
1	2.995	0	0	3.013	0	0
2	3.006	2.88	3.01	2.994	2.16	3.01
3	2.999	2.80	3.01	3.009	2.16	3.01

The surviving medium of Exp. 1 was 15 cc. of modified Ringer's solution, those of Exp. 2 and 3 were both 15 cc. of modified Ringer's solution and 1 cc. of glycocyamine (3.01 mg./cc.) solution.

(This experiment was carried out before the period of incubation was fixed for 3 hours).

IV. THE PERFUSION EXPERIMENT WITH RABBIT LIVER.

Although the investigation of Beard and Boggess (1935), suggests the creatine-body formation from protein or amino-acid, no increase of the total creatinine-equivalent is found under the above mentioned experimental condition. To study the mechanism under a more natural condition than in the above case, the perfusing experiment of the liver in situ was undertaken with and without the addition of amino-acids, glycocyamine, guanidine and thyroxine to the perfusing fluid (see Table VI_a).

1. Perfusing apparatus: Slightly modified apparatus of Yamakawa and Mizuki (1918) was used. Some modification was made on the pump of their apparatus to fit it for any diameter of rubber tube. Moreover, I employed about a 30 cm. length oxygenating vessel to avoid the overflowing of foam produced by oxygenation under stirring and to insert the oxygenating vessel near the bottom of the thermostat to facilitate the flowing back of the liquid from the perfused liver.

2. Animal: Male rabbits weighing 3000–2700 gm. were used after 2 days of starvation.

3. Preparation of perfusing fluid: The blood was shed from the carotid artery by means of cannula into a 200 cc. Erlenmeyer's flask, into which 50 cc. of modified Ringer's $solution_2$ (see Table III_a) was previously measured. The diluted blood was defibrinated by stirring with a glass-rod for about 20 minutes and filtered through clean cloth into the 250 cc. measuring cylinder and measured. The volume of the original defibrinated blood is determinad by the deduction of 50 cc. from this volume. To this diluted blood a certain amount of modified Ringer's $solution_2$, or modified Ringer's $solution_2$, together with 5 cc. or 10 cc. of solution containing amino-acids, glycocyamine, guanidine, and thyroxine separately or together (see Table VI_a). The volume of modified Ringer's $solution_2$ shown on the Table VI_a is calculated with the addition of a volume (2–3 cc.) of carbonate-free Ringer's solution usually remaining in cannulae after washing the liver.

4. Operation of perfused liver: As soon as the blood was shed, the abdomen and chest of the animal were cut open. After tying off the ductus choledochus, the hepatic artery and the branches of the portal vein, avoiding lesion of the liver, a cannula was inserted into the portal vein and about 300 cc. of

TABLE VIa.

The composition of the perfusing fluids used for the experiments described on Table VIb.

Symbol	Composition	Total volume
P_1	Blood 55 cc.+M. R. S.$_2$ 138 cc.	193 cc.
P_2	„ 78 cc.+ „ 125 cc.+thyr. (0.7 mg.) in 5 cc.	208
P_3	„ 62 cc.+ „ 127 cc.+thyr. (0.7 mg.) in 5 cc.+ gcya.-glyc.-arg. (16 mg., 43 mg., 43 mg) in 5 cc.	199
P_4	Blood 50 cc.+M. R. S.$_2$ 138 cc.+gcya.-glyc.-arg. (16 mg., 43 mg., 43 mg.) in 5 cc.	193
P_5	Blood 68 cc.+M. R. S.$_2$ 114 cc.+thyr. (0.6 mg.) in 5 cc.+ glyc. (100 mg) in 5 cc.	192
P_6	Blood 60 cc.+M. R. S.$_2$+122 cc.+thyr. (0.6 mg.) in 5 cc.+ arg. (102 mg.) in 5 cc.	„
P_7	Blood 53 cc.+M. R. S.$_2$ 130 cc.+thyr. (0.7 mg.) in 5 cc.+ gcya. (16 mg.) in 5 cc.	193
P_8	Blood 60 cc.+M. R. S.$_2$ 122 cc.+thyr. (0.65 mg.) in 5 cc.+ gcya. (22 mg.) in 5 cc.	192
P_9	Blood 54 cc.+M. R. S.$_2$ 128 cc.+thyr. (0.7 mg.) in 5 cc.+ gcya. (16 mg.) in 5 cc.	„
P_{10}	Blood 63 cc.+R. M. S.$_2$ 125 cc.+gcya. (16 mg.) in 5 cc.	193
P_{11}	„ 53 cc.+ „ 134 cc.+gcya. (22 mg.) in 5 cc.	192
P_{12}	„ 59 cc.+ „ 128 cc.+gcya. (16 mg.) in 5 cc.	„
P_{13}	„ 55 cc.+ „ 128 cc.+thyr. (0.7 mg.) in 5 cc.+ glyc.-arg. (45 mg., 41 mg.) in 5 cc.	193
P_{14}	Blood 63 cc.+M. R. S.$_2$ 119 cc.+thyr. (0.6 mg.) in 5 cc.+ glyc.-arg. (50 mg., 51 mg.) in 5 cc.	192
P_{15}	Blood 53 cc.+M. R. S.$_2$ 129 cc.+thyr. (0.7 mg.) in 5 cc.+ glyc.-arg. (46 mg., 44 mg.) in 5 cc.	„
P_{16}	Blood 60 cc.+M. R. S.$_2$ 122 cc.+thyr. (0.7 mg.) in 5 cc.+ glyc.-arg. (45 mg., 44 mg.) in 5 cc.	„
P_{17}	Blood 68 cc.+M. R. S.$_2$ 119 cc.+glyc.-arg. (45 mg., 41 mg.) in 5 cc.	„
P_{18}	Blood 63 cc.+M. R. S.$_2$ 128 cc.+glyc.-arg. (45 mg., 44 mg.) in 5 cc.	196
P_{19}	Blood 48 cc.+M. R. S.$_2$ 134 cc.+thyr. (0.5 mg.) in 5 cc.+ ala.-arg. (57 mg., 53 mg.) in 5 cc.	192
P_{20}	Blood 60 cc.+M. R. S.$_2$ 122 cc.+thyr. (0.7 mg.) in 5 cc.+ ala.-arg. (51 mg., 44 mg.) in 5 cc.	„
P_{21}	Blood 72 cc.+M. R. S.$_2$ 120 cc.+thyr. (0.6 mg.) in 5 cc.+ glyc.-gua. (30 mg., 36 mg.) in 5 cc.	202
P_{22}	Blood 76 cc.+M. R. S.$_2$ 106 cc.+thyr. (0.75 mg.) in 5cc.+ lys.-arg. (97 mg., 54 mg.) in 5 cc. +isot. $NaHCO_3$ 2 cc.	194
P_{23}	Blood 62 cc.+M. R. S.$_2$ 130 cc.+thyr. (0.75 mg.) in 5 cc.+ lys.-glyc.-arg. (57 mg., 55 mg., 52 mg.) in 5 cc.	202
P_{24}	Blood 59 cc.+M. R. S.$_2$ 123 cc.+thyr. (0.75 mg.) in 5 cc.+ lys.-glyc.-arg. (58 mg., 45 mg., 50 mg.) in 5 cc.	192
P_{25}	Blood 53 cc.+M. R. S.$_2$ 134 cc.+gcya. (16 mg.) in 5 cc.	„

Blood = Defibrinated blood.
M. R. S.$_2$ = Modified Ringer's solution$_2$ (see Table IIIa).
Thyr. = Thyroxine: snspended in 5 cc. of isot. $NaHCO_3$.
Gcya. = Glycocyamine.
Glyc. = Glycocoll.
Arg. = Arginine carbonate.
Ala. = d-Alanine.
Gua. = Guanidine carbonate.
Lys. = Lysine dihydrochloride.
Gcya.-glyc.-arg. (16 mg., 43 mg., 43 mg.) in 5 cc. = Glyocyamine, glycocoll and arginine carbonate mixed solution (respectively 16 mg., 43 mg., and 43 mg. were dissolved in 5 cc. of water). This applies to the corresponding parts. All the solutions of these substances were water solutions, but when glycocyamine alone was dissolved it was dissolved in 5 cc. of 0.9% NaCl.

warm (37° C.) carbonate-free Ringer's solution were slowly infused to wash the liver thoroughly, with the incisions at the inferior vena cava under the heart and the kidney. When the outcoming fluid became colorless, the vena cava was tied up above the right suprarenal gland, while the second cannula was inserted from the incised point of the vena cava near the heart directly above the diaphragm. Some amount of carbonate-free Ringer's solution was again infused to acertain a smooth passage. The liver together with the animal, without removing the cannulae, was held in the warming chest until the perfusion was started.

5. Procedure of perfusion: The greater part of the perfusing apparatus except the pump was set in a thermostat at 39°C. The total volume of the perfusing fluid was circulated through the perfusing apparatus alone for about 3 minutes to remove the air bubbles adhering to the rubber tube, etc. And then the rubber tube of the perfusing apparatus was connected with the cannulae which were inserted into the portal vein and the vena cava, preventing air bubbles from entering the circuit. After circulating the perfusing fluid through the liver for 7–10 minutes, the pump was stopped for a while, and a portion of the liver weighing about 3 gm. was cut off, preventing bleeding from the remaining main part of the liver, by the use of Kocher's clamp. After the fluid adhering to the excised liver was wiped

off with dry clean cloth, the liver was transferred to the weighing bottle. This portion of the liver was used for the determination of the total creatinine-equivalent in the perfusing liver previous to perfusion. At the same time 20 cc. of the perfusing fluid of the oxygenator were pipetted into a 50 cc. volumetric flask to estimate the total creatinine-equivalent prior to perfusion. Then the perfusion was continued for 2 hours by means of the pump under 10–13 mm Hg pressure. After 2 hours of perfusion, the pump was stopped again, and another portion of the perfused liver was cut off and transferred to the weighing bottle in the same manner as described above to estimate the total creatinine-equivalent after the perfusion. 20 cc. of the perfusing fluid were again measured into another 50 cc. volumetric flask to determine the total creatinine-equivalent after 2 hours of perfusion. Furthermore, the amounts of the remaining perfused liver and the perfusing fluid were determined. The sum of the amount of last remaining liver and that of the cut off portion for the estimation of the total creatinine-equivalent after 2 hours perfusion, is the true amount of the perfused liver. This amount is shown on Table VI$_b$ as the amount of the perfused liver. As the perfusing fluid leaks out more or less during perfusion, the true volume of the perfusing fluid is to be taken as the mean of the volume of the total perfusing fluid deducting 20 cc. which were used for the determination of the total creatinine-equivalent before perfusion, and the volume of the remaining perfusing fluid added with the amount of the perfusing fluid which was used for the determination of the total creatinine-equivalent after 2 hours of perfusion. For the measurment of the remaining amount of the perfusing fluid, the amount remaining in the perfusing apparatus (usually 8 cc.) was taken into consideration.

6. Determination of total creatinine-equivalent of perfused liver and perfusing fluid: a). Perfused liver. The total creatinine-equivalent of the perfused liver was determined as mentioned before. b). Perfusing fluid. As the total creatine-body was found fully extracted from nonhemolysed fluid with trich-

TABLE

The effect of the perfusion of rabbit liver in situ on the total creatinine-

Exp. No.	Rabbit No.	Symbol of the perfusing fluid (See Tab. VIa)	Main added substances to the perfusing fluid (See Table VIa)	Volume of the perfusing fluid. cc.	Amount of the perfused liver. gm.
XI	22	P_1	—	169	45.9
XII	23	P_2	Thyr.	177	71.4
XIII	24	P_3	Thyr., gcya., glyc., arg.	174	57.8
XIV	25	P_4	Gcya., glyc., arg.	167	56.8
XV	26	P_5	Tbyr., glyc.	171	56.4
XVI	27	P_6	Thyr., arg.	167	52.2
XVII a[1]	28	P_7	Thyr., gcya.	169	42.5
b	29	P_8	,,	168	50.2
c	30	P_9	,,	164	39.9
XVIII a	31	P_{10}	Gcya.	171	41.7
b	32	P_{11}	,,	155	42.9
c	33	P_{12}	,,	166	56.3
XIX a	34	P_{13}	Thyr., glyc., arg.	172	54.0
b	35	P_{14}	,,	168	45.9
c	36	P_{15}	,,	170	50.0
d	37	P_{16}	,,	171	52.1
XX a	38	P_{17}	Glyc., arg.	170	48.8
b	39	P_{18}	,,	176	62.5
XXI a	40	P_{19}	Thyr., arg., ala.	167	53.7
b	41	P_{20}	,,	170	52.0
XXII	42	P_{21}	Thyr., gua., glyc.	178	57.8
XXIII[2]	43	P_{22}	Thyr., lys, arg.	157	71.7
XXIV a	44	P_{23}	Thyr,. lys., glyc., arg.	182	55.9
b	45	P_{24}	,,	171	59.6
XXV a[3] b[4]	46	P_{25}	Gcya.	169	39.3

[1] Period of perfusion was 2 hrs. and 20 minutes.

[2] Period of perfusion was 1.5 hrs.

[3] Test filtrate was heated at 120°C. for 45 minutes in an autoclave as usual.

[4] Test filtrate was heated at 160°C. for 45 minutes in an autoclave.

VIb

equivalent in the case of the addition of various substances.
(T. C. E. = Total creatinine-equivalent)

Before perfusion			After 2 hours perfusion				
T. C. E. (mg.)			T. C. E. (mg.)			Increased or decreased T. C. E. in the sum (mg.)	Increased or decreased T. C. E. in the sum per 100 gm. liver (mg. %)
Perfusing fluid	Perfused liver	Sum	Perfusing fluid	Perfused liver	Sum		
2.22	5.97	8.19	4.04	4.22	8.26	+0.07	+ 0.15
2.65	5.79	8.44	4.16	4.42	8.58	+0.14	+ 0.20
2.82	4.53	7.35	3.15	7.77	10.92	+3.57	+ 6.17
3.31	4.65	7.96	4.14	7.92	12.06	+4.10	+ 7.22
2.22	3.77	5.99	2.96	3.04	6.00	+0.01	+ 0.02
2.16	5.95	8.11	3.64	4.21	7.85	−0.26	− 0.50
2.41	2.29	4.70	3.81	5.13	8.94	+4.24	+ 9.99
4.12	9.60	13.72	6.86	9.22	16.08	+2.36	+ 4.70
3.42	3.99	7.41	4.87	5.06	9.93	+2.52	+ 6.32
3.44	6.50	9.94	5.09	10.61	15.70	+5.76	+13.80
2.45	4.48	6.93	3.38	5.41	8.79	+1.86	+ 4.33
3.99	6.65	10.64	5.29	11.22	16.51	+5.87	+10.43
2.31	3.32	5.63	3.25	3.64	6.89	+1.26	+ 2.33
1.66	2.19	3.85	2.25	2.40	4.65	+0.80	+ 1.74
2.26	5.08	7.34	3.54	4.14	7.68	+0.34	+ 0.68
2.70	3.85	6.55	4.06	4.50	8.56	+2.01	+ 3.86
1.55	2.85	4.40	2.14	2.27	4.41	+0.01	+ 0.02
2.75	4.13	6.88	3.52	3.76	7.28	+0.40	+ 0.64
1.89	5.28	7.17	3.34	4.62	7.96	+0.79	+ 1.47
2.08	3.15	5.23	2.97	2.99	5.96	+0.73	+ 1.40
2.27	5.42	7.69	3.88	3.73	7.61	−0.08	− 0.14
2.64	5.77	8.41	3.52	4.98	8.50	+0.09	+ 0.12
2.77	6.50	9.27	4.64	4.86	9.50	+0.23	+ 0.41
2.77	5.97	8.74	3.71	4.99	8.70	−0.04	− 0.07
2.79	4.13	6.92	3.92	4.65	8.57	+1.65	+ 4.20
4.68	4.85	9.53	4.61	6.37	10.98	+1.45	+ 3.69

Thyr. = Thyroxine.
Gcya. = Glycocyamine.
Glyc. = Glycocoll.
Arg. = Arginine.

Ala. = Alanine.
Gua. = Guanidine.
Lys. = Lysine.

loracetic acid, the extraction of the creatine-body was made by adding gradually 5 cc. of trichloracetic acid solution under shaking to the 50 cc. volumetric flask containing 20 cc. of the perfusing fluid. The flask was filled to the mark with water and thoroughly mixed. After standing for one hour or longer it was filtered. The succeeding procedures are performed just the same as in the case of liver.

7. Calculation of total creatinine-equivalent before and after perfusion: From the results of determination with liver and perfusing fluid, the total amount of creatine-body in the real perfused liver and the real perfusing fluid were calculated. The comparison of the sum of these two values before and after perfusion indicates to us whether an increase or decrease of creatine-body occured during perfusion.

8. Result of experiment: As can be seen from the table, the addition of glycocyamine to the perfusing fluid increases equally the total creatinine-equivalent after perfusion in all cases with or without thyroxine or other substances. On the other hand, it was found out that though glycocyamine did not give any colour production with alkaline picrate solution, it was completely tranformed to glycocyamidine to give full colour production, when for instance 10 cc. of glycocyamine solution containing 0.5 mg. were heated at 160°C. for 45 minutes with 1 cc. of 2N HCl in an autoclave, while when heated at 120° C. for 45 minutes about 1/4 or less of glycocyamine was transformed to glycocyamidine. Therefore, the possibility might exist that the glycocyamine was transformed merely to glycocyamidine by the action of the liver to give rise to the increase of colour. Assuming this to be possible, a part of the filtrate was heated at 160°C. for 45 minutes with 2N HCl in an autoclave to determine the amount of remainng glycocyamine by transforming it to glycocyamidine and to compare the value of the colour increase as glycocyamidine. In spite of these considerations the increase of the total creatinine-equivalent after perfusion was really observed (Exp. XXV_b). When arginine and glycocoll together with thyroxine are added to the perfusing fluid, the

total creatinine-equivalent increases also as shown in the table (Exp. XIX), though the increase is relatively small. When alanine was added to the perfusing fluid instead of glycocoll, the increase is still smaller. (Exp. XXI). The omission of thyroxine in these cases or the addition of lysine to the mixture containing amino-acids and thyroxine causes no increase after perfusion (Exp. XX and XXIV). Furthermore, as can be seen from the table, unless the increase of the total creatinine-equivalent after perfusion is relatively great, the increase in the liver alone should not be recognized.

V. DISCUSSION.

Inouye (1912) reported that the total creatinine increased in the perfusion experiment on liver, in which arginine alone was added to the perfusing fluid. Beard and Boggess (1936) reported that parenteral injection of glycocyamine, arginine, glycine, alanine etc. gave an increase in muscle creatine, and concluded from this result that creatine formation took place chiefly in the muscles.

According to Bodansky (1935), the administation of arginine and other amino-acids, except glycine and alanine, produced no effect on the excretion of creatine or creatinine in rats. The administration of glycine on the other hand did not influence the excretion of creatinine, but definitely increased the output of creatine. Similar, though less striking or constant changes, were obtained with alanine. The increased elimination of creatine following the administration of guanidine-acetic acid suggests the probable conversion of the latter into creatine. Furthermore, according to his paper in 1936, guanidoacetic acid was not found in the heart, muscle, and liver of normal rats, but traces were present in the kidneys and alimentary tract, including its contents. At intervals following the administration of of guanidoacetic acid, its distribution was determined. Of the amount absorbed, considerable quantities appeared in the liver and kidneys. Although its presence in muscle and heart was definite, the concentration was insufficient for quantitative estimation. As much as 50 per cent was excreted in the urine unchanged. These findings were accompanied by a definite increase of creatine in the kidneys, suggesting that methylation of the guanidoacetic acid may have occurred in the kidneys. However, it is not assumed that muscle may not be the principal site of guanidoacetic acid methylation in normal metabolism. In view of the occurence of guanidoacetic acid in large amounts in the liver and the failure to show an increase in creatine, it is surmised that the liver plays an insignificant rôle, if any, in creatine production. The results of his study furnish additional evidence for the conversion of glycocyamine into creatine. In this same paper he does not support the contention that glycine is a precursor of creatine as no change appears anywhere in the glycine-fed rat.

Our above mentioned experiments on the liver suggest to us a conclusion that at least a part of the glycocyamine seems to be transformed to creatine in the liver. However, there is also possibility enough that glycocyamine might be converted into glycocyamidine during the experiment. The tendency of glycocyamine stagnation in the liver reported by Bodansky could be observed also in my prefusion experiment (Tab. VI_b, Exp. $XXV_{a,\ b}$). Throughout all our experiments the increase of total creatinine-equivalent in the liver alone was comparatively small. This fact may indicate that, as soon as the glycocyamine changes into creatine, the formed creatine may be easily transported to some other organs or tissues through the hepatic vein.

Bergmann and Zervas (1927) synthesized artificially diacetyl-glycocyamineethylester and monoacetyl-glycocyamidine from the mixture of triacetyl-anhydro-arginine and glycocollester in water-free ether and they discussed the mechanism of glycocyamine formation. Our recognization of the total creatinine-equivalent increase in perfusion experiments on liver under the simultaneous addition of arginine, glycocoll, and thyroxine, though such an increase was missed in the incubating experiment with the addition of these substances, may have some relation with the experiment of Bergmann and Zervas. On the other hand, further study of the production of chromogenic substances, except creatine-body, must be extended. I am performing some further experiments on these questions in detail at present.

SUMMARY

1. A method of determination of total creatinine was worked out.

2. The influence of the various substances on the amount of total creatinine-equivalent after autolysis and perfusion of rabbit liver was studied by this method.

3. The addition of the glycocyamine increased the total creatinine-equivalent in the incubation and perfusion experiments of the liver.

4. The simultaneous addition of arginine, glycine and thyroxine to the perfusing fluid, caused the increase of the total creatinine-equivalent in the perfusion experiment on liver.

I wish to express my sincere thanks to Prof. S. Kakiuchi for his kind advice throughout the course of the investigation.

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INDEX TO VOLUME 25.

A

Abe, Yosimi. A colorimetric microdetermination of dexoxycholic and cholic acid in bile. 181.

Acidosis, The gaseous content of arterial, cutaneous and venous blood in the normal state, —— and alkalosis. 89.

Akasi, Syûzo. Studies über die Konstitution des Octopins, eines stickstoffhaltigen Körpers in den Octopodenmuskeln. I. Mitteilung: Eigenschaften und Abbau des Octopins. 261.
II. Mitteilung: Synthetische Versuche. 281.
III. Mitteilung: Stereochemische Untersuchung mittels der Drehkurvenmethode nach Lutz. 291.

Akizuki, Hiroshige. Studien über das Histozym. 43.

Akutsu, Nankoo and Fujii, Nobuzoo. A new titration method for sugar determination. 237.

Alkalosis, The gaseous content of arterial, cutaneous and venous blood in the normal state, acidosis and ——. 89.

Amino acids, The reaction of —— ——, peptides and related substances with sugars. I. 607.

Aminooxypropansulfonsäure, Über die Derivate der ——. 375.

Antigenic substance, On the —— —— contaminated within phospholipin. 595.

Ariyama, N. and Kitasato, T. The disintegration of methylated glucoses in alkaline medium. 357.

Arterial blood, Variations in the alveolar gaseous pressures as well as the gaseous contents of —— —— and its pH in man at rest. 453.

Atmungsfähigkeit, Eisen-Porphyringehalt und —— der verschieden genährten Hefen. 627.

Azorubine S, On the liver function, with special reference to the excretions of ——, congo red and santonin dyes. 533.

B

Bacillus coli communis, Bildung der 7-Oxy-3.12-Diketocholansäure aus Dehydrocholsäure durch —— —— ——. 61.

Bacilles tuberculeux, Sur le metabolisme de la cholestérine chez les cobayes inocules avec des —— ——. 657.

Bezssonoff's reacting substance, On —— ——s, and on its identity to Vitamin C. 219.

Bile, A colorimetric microdetermination of desoxycholic and cholic acid in ——. 181.

Biuretreaktion, Beiträge zur Kenntnis der ——. 375.

Blood gas studies performed with a new micro blood gas apparatus. I. 79, 89.

Blood pH, Studies on the —— —— estimated by the glass electrode method. 493.

Blut, Über den gärbaren, hydrolysierbaren Zucker im ——, insbesondere dessen Mikrobestimmung. 1.

Blut, Über die Phosphorverbindungen des ——es verschiedener Fischarten. 393.

Blut, Über das Verhalten des Cholesterins und seiner Ester im ——e bei verschiedenen Leberkrankheiten. 461.

Blutgalaktose, Über die Bestimmungsthode der ——. 11.

C

Canalin, A die-amino acid, canavanin, and a monoamino acid. 23.

Canavanin, A di-amino acid,——, and a mono-amino acid, canalin. 23.

Cholansäure, Beitrag zur Kenntnis der 3-Oxy-6- Ketoallocholansäure und Synthese der α-3.6-Dioxyallo-——. 157.

Cholesterin, Über das Verhalten des ——s und seiner ester im Blute bei verschiedenen Leberkrankheiten. 461.

Cholestérine, Sur la teneur en —— des ongles de quelques animaux. 109.

Cholestérine, Sur le metabolisme de la —— chez les cobayes inocules avec des bacilles tuberculeux. 657.

Cholic acid, A colorimetric microdetermination of desoxycholic and —— —— in bile. 181.

Cholsäure, Ausscheidung des Magnesium unter Einfluss von —— bei normalen sowie bei splanchnikotonierten Kaninchen. 435.

Cobaye, Sur le metabolisme de la cholestérine chez les ——s inocules, avec des bacilles tuberculeux. 657.

Colorimetric microdetermination, A —— —— of desoxycholic and cholic acid in bile. 181.

Congo red, On the liver function, with special reference to the excretions of azorubine S, —— —— and santonin dyes. 533.

Creatine formation, Studies on the mechanism of creatine formation. 701.

D

Dehydrocholsäure, Bildung der 7-Oxy-3.12-Diketo-Cholansäure aus —— durch Bacillus coli communis. 61.

Dental caries, Some possible factors concerning the development of —— ——. 671.

Desoxycholic acid, A colorimetric microdetermination of —— and cholic acid in bile. 181.

di-amino acid, A ——, canavanin, and a mono-amino acid, canalin. 23.

Diketocholansäure, Bildung der 7-Oxy-3-12 —— aus Dehydrocholsäure durch Bacillus coli communis. 61.

E

Eisen-porphyrin, Studien über Bedeutungs des —— im Zellstoffwechsel. I. 627.

Erde, Studium über die Fettstoffe der Säurefestbazillen in der ——. 113.

Ergosterol, The micro determination of the iodine number of sterols in the tissues with special reference to ——. 417.

Extinktionskoefficienten, Über die photoelektrische Bestimmung der —— der Lösungen. 193.

F

Fettsäure, Über den Umsatz der ——n

in der Leber. 141.

Fettstoffe, Studium über die —— der Säurefestbazillen in der Erde. 113.

Fetus, Über den Einfluss der Nahrungsfette auf die Eigenschaften der Phosphatid-Fettsäure des ——. 579.

Frog muscle, The effect of ultraviolet- and roentgenrays on the redox-potential of the —— —— in vivo. 207.

FUJII, Nobuzoo and AKUTSU, Nankoo. A new tritration method for sugar determination. 237.

FUJIMOTO, T., SHOJI, R., YOSHIMURA, H. and SAITO, K. Variations in the alverolar gaseous pressures as well as the gaseous contents of arterial blood and its pH in man at rest. 453.

FUJIMOTO, Tomitaro and YOSHIMURA, Hisato. Is the hydrogen gas electrode not applicable to the determination of the pH of oxygenated blood? 493.

FUJIMURA, Shiro. On the antigenic substance contaminated within phospholipin. 595.

FUJITA, Akiji und SAKAMOTO, Toshimasa. Über die photoelektrische Bestimmung der Extinktionskoefficienten der Lösungen. 193.

FUKUI, Tomoyasu. Bildung der 7-Oxy-3.12-Diketo-cholansäure aus Dehydrocholsäure durch Bacillus coli communis. 61.

G

Gallensäure, Magnesiumstoffwechsel und ——. 435.

Gaseous content, Variations in the alveolar gaseous pressures as well as the —— ——s of arterial blood and its pH in man at rest. 453.

Gaseous pressure, Variations in the alveolar —— ——s as well as the gaseous contents of arterial blood and its pH in man at rest. 453.

Glass electrode method, Studies on the blood pH estimated by the —— —— ——. 493.

Gliadin, Partielle Spaltung des ——s 307.

α-Guanidoglutarsäure, Über ——. 339.

H

Harington and van Slyke. A modification of the —— —— —— extraction chamber. 79.

Hefe, Eisen-Porphyringehalt und Atmungsfähigkeit der verschieden genährten ——. 627.

Hématies, Changement caractéristique de la vitesse de la sédimentation des —— dans les solutions de concentration oligolytique. 555.

Hemolysin, Effectiveness of stroma protein on the production of ——. 691.

HIROHARA, Ryozo. Studies on protamine. 519.

Histozym, Studies über das ——. 43.

HOTTA, Kazuo et TAKAGI, Kenzo. sur la teneur en cholestérine des ongles de quelque animaux. 109.

Hydrogen gas electrode, Is the —— —— —— not applicable of oxygenated blood. 493.

Hydrogen peroxide electrode, On the —— —— ——. 573.

Hyperglykämie. über die —— nach der Exstirpation der Nebenniere. 133.

I

IMAI, Toshitake. Chemistry of vitamin

B_1. 95.

Indigo-carmine, On the renal function, with special reference to the excretion of —— and phenolphtalein. 525.

Iodine number, The micro determination of the —— of sterols in the tissues with special reference to ergosterol. 417.

Iseki, Toshinori. Synthesen der γ-Methylamino-β-Oxy-Butersäure und γ-Methylguanido-β-Oxybuttersäure. 549.

ITO, N. und Y. KOTAKE (jr.) Studien über den intermediären Stoffwechsel des Tryptophans. XXV. Isolierung des d-Kynurenins. 71.

ITOH, Ryoji. Studies on lipase. II Report. 167.

ITZIOKA, Fuyutaro. Über das Trypsinpepton (Trypton). 329.

IWASAKI, Yoshio. Studies on lipase. III. Report. Effect of overian follicular hormone on the pancreatic lipase. 177.

K

KAMEDA, Hiroo. Studium über die Fettstoffe der Säurefestbazillen in der Erde. 113.

Kaninchen, Ausscheidung des Magnesium unter Einfluss von Cholsäure bei normalen sowie bei splanchnikotomierten ——. 435.

KAZUNO, Taro. Über ein neues Sterin (Sapogenol) aus "Shoyu-Öl" (I). 251.

KAZUNO, Taro u. SHIMIZU, Tayei. Untersuchung der Krötengalle. VI. Konstitution der Trioxyisosterocholensäure. 245.

Ketoallocholansäure, Beitrag zur Kenntnis der 3-Oxy-6- —— und Synthese der α-3,6-Dioxy-allo-Cholansäure. 157.

KITAGAWA, Matsunosuke. A di-amino acid, canavanin, and a mono-amino acid, canalin. 23.

KITASATO, T. and ARIYAMA, N. The disintegration of methylated glucoses in alkaline medium. 357.

KODAMA, Keizo and ONIZUKA, Toshio. On the hydrogen peroxide electrode. 573.

KOMITA, Yasuo. Studies on "Nucleotide-N-Ribosidase." 405.

KOTAKE, Y. (jr.) und N. ITO. Studien über den intermediären Stoffwechsel des Tryptophans. XXV. Isolierung des d-Kynurenins. 71.

KOYAMA, Kazuo. Über den Umsatz der Fettsäuren in der Leber. 141.

Kreatin, Betrachtung über die möglichen Vorstufen des ——s. 339.

Krötengalle, Untersuchung der ——. 245.

KUNISHIGE, Taro. Untersuchungen über den partiellen Abbau der Proteine. II. Mitteilung. Partielle Spaltung des Gliadins. 307.

KURODA, Kaitiro et NAKAMURA, Hirosi. Changement caractéristique de la vitesse de la sédimentation des hématies dans les solutions de concentration oligolytique. 555.

KUSUI, Kenzo. Über das Verhalten des Cholesterins und seiner Ester im Blute bei verschiedenen Leberkrankheiten. 461.

d-Kynurenin, Isolierung des ——s. 71.

L

Leber, Über den Umsatz der Fettsäuren in der ——. 141.

Leberkrankheit, Über das Verhalten des Cholesterins und seiner Ester im Blute bei verscheidenen ——. 461.

Lipase, Studies on ——. 167, 177.

Liver, On the —— function with special reference to the excretions of azorubine S, congo red and Santonin dyes. 535.

M

Magnesiumstoffwechsel und Gallensäure. 435.

MASAMUNE, Hajime. On detoxication of naphthalene ingested, with special reference to a new uronic acid excreted. Preliminary Report. 299.

γ-Methylamino, Synthese der —— - β-Oxy-Buttersäure und -Methylguanido-β-Oxybuttersäure. 549.

Methylated glucoses, The disintegration of —— —— in alkaline medium. 357.

γ-Methylguanido, Synthese der γ-Methylamino - β - Oxy - Buttersäure und —— - β-Oxybuttersäure. 549.

Micro determination, The —— —— of the iodine number of sterols in the tissues with special reference to ergosterol. 417.

Mikrobestimmung, Über den gärbaren, hydrolysierbaren Zucker im Blut, insbesondere dessen ——. 1.

MIURA, Kichihei, Über den Einfluss der Nahrungsfette auf die Eigenschaften der Phosphatid-Fettsäure des Fetus. 579.

MIZUMA, Morisaburo. Some possible factors concerning the development of dental caries. 671.

Mono-Amino acid, A di-amino acid, canavanin, and a —— ——, canalin. 23.

N

Nahrungsfette, Über den Einffuss der —— auf die Eigenschaften der Phosphatid-Fettsäure des Fetus. 579.

NAKAMURA, Hirosi et KURODA, Kaitirô. Changement caractéristique de la vitesse de la sédimentation des hématies dans les solutions de concentration olygolytique. 555.

Naphthalene, On detoxication of —— ingested, with special reference to a new uronic acid excreted. 299.

Nebenniere, Über die Hyperglykämie nach der Exstirpation der ——. 133.

New-born infant, Studies on the changes in the physiological condition of the —— —— —— ——. 525, 535.

Nucleotide-N-Ribosidase, Studies on ——. 405.

O

Octopin, Studien über die Konstitution des —— eines stickstoffhaltigen Körpers in den Octopodenmuskeln. 261.

Octopodenmuskeln, Studien über die Konstitution des Octopins, eines stickstoffhaltigen Körpers in den ——. 261.

OGAWA, Tetsutaro. Über die Phosphorverbindungen des Blutes verschiedener Fischarten. 393.

OHTA, Reishi. Über den gärbaren, hydrolysierbaren Zucker im Blut, insbesondere dessen Mikrobestimmung. 1.

ONIZUKA, Toshio and KODAMA, Keizo. On the hydrogen peroxide electrode. 573.

Ongle, Sur la teneur en Cholestérine des ——s de quelques animaux. 109.

Organismus, Über die Oxydation des ω-Oxymethylfurfurols im ——.

487.
Ovarian follicular hormone, Effect of —— —— —— on the pancreatic lipase. 177.
β-Oxy-Buttersäure, Synthese der γ-Methylamino- —— —— und γ-Methylguanido-B-Oxybuttersäure. 549.
Oxydation, Über die —— des ω-Oxymethylfurfurols im organismus. 487.
Oxygenated blood, Is the hydrogen gas electrode not applicable to the determination of the pH of —— ——. 493.
ω-Oxymethylfurfurols, Über die Oxydation des —— im Organismus. 487.
Ozaki, Tohl. Über die Hyperglykämie nach der Exstirpation der Nebenniere. 133.

P

Pancreatic lipase, Effect of ovarian follicular hormone on the —— ——. 177.
Peptides, The reaction of amino acids, ——. and related substances with sugar. 607.
Phenolsulphonephthalein, On the renal function with special reference to the excretion of indigo-carmine and ——. 525.
Phosphatid, Eine neue Methode zur Bestimmung von ——. 151.
Phosphatid-Fettsäure, Über den Einfluss der Nahrungsfette auf die Eigenschaften der —— des Fetus. 579.
Phospholipin, On the antigenic substance contaminated within phospholipin. 595.
Phosphorverbindungen, Über die —— des Blutes verschiedener Fischarten. 393.
Photoelektrische Bestimmung, Über —— —— der Extinktionskoefficienten der Lösungen. 193.
Protamine, Studies on ——. 519.
Proteine, Untersuchung über den partiellen Abbau der ——. 307.

R

Redox-potential of the frog muscle in vivo. 207.
Renal function, On the —— —— with special reference to the excretion of indigo-carmine and phenolsulphonephthalein. 525.
Roentgen-rays, The effect of ultraviolet- and —— —— on the redox-potential of the frog muscle in vivo. 207.

S

Saito, Koichiro. A modification of the Harington and van Slyke extraction chamber. 79.
Saito, Koichiro. The gaseous content of arterial, cutaneous and venous blood in the normal state, acidosis and alkalosis. 89.
Saito, K., Shoji, R., Yoshimura, H. and Tujimoto, T. Variations in the alveolar gaseous pressures as well as the gaseous contents of arterial blood and its pH in man at rest. 453.
Sakamoto, Toshimasa und Fujita, Akiji. Über die photoelektrische Bestimmung der Extinktionskoefficienten der Lösungen. 193.
Sapogenol, Über ein neues Sterin —— aus "Shoyu-Öl". 251.
Sang, Sur le taux des cholestérines libres et éthérifiées dans le —— total du cobaye normal et du

cobaye tuberculeux. 657.

Santonin dyes, On the liver function, with special reference to the excretions of azorubine S, congo red and —— ——. 533.

Säurefestbazillen, Studium über die Fettstoffe der —— in der Erde. 113.

Scurvy, Studies in experimental ——. 219.

SENDJU, Yuzo. Über ω-Oxymethylfurfurols in Organismus. 487.

SHIBUYA, Shin-ichi. Studies on the mechanism of creatine formation. 701.

SHIGA, Naoshi. The reaction of amino acids, peptides and related substances with sugars. I. 607.

SHIMAZONO, Norio. Effectiveness of stroma protein on the production of Hemolysin. 691.

SHIMIZU, Tayei u. KAZUNO, Taro. Untersuchung der Krötengalle. VI. Konstitution der Trioxy-isosterocholensäure. 245.

SHOJI, Tadashi. Studies on the changes in the physiological condition of the new-born infant. III. On the renal function, with special reference to the excretion of indigo-carmine and phenolsulphonephthalein. 525.

IV. On the liver function, with special reference to the excretions of azorubine S, congo red and santonin dyes. 533.

SHOJI, R., YOSHIMURA, H., SAITO, K. and TUJIMOTO, T. Variations in the alveolar gaseous pressures as well as the gaseous contents of arterial blood and its pH in man at rest. 453.

Shoyu-Öl, Über ein neues Sterin (Sapogenol) as ——. 251.

Splanchnikotomierten Kaninchen, Ausscheidung des Magnesium unter Einfluss von Cholsäure bei normalen sowie bei —— ——. 435.

Sterin, Über ein neues —— (Sapogenol) aus "Shoyu-Öl". 251.

Sterol, The micro determination of the iodine number of ——s in the tissues with special reference to ergosterol. 417.

Stoffwechsel, Studien über den intermediären —— des Tryptophans. 71.

Stroma protein, Effectiveness of —— —— on the production of hemolysin. 691.

Sugar, The reaction of amino acids, peptides and related substances with ——s. 607.

Sugar determination, A new titration method for —— ——. 237.

SUGAWARA, Sho. Über die Bestimmungsmethode der Blutgalaktose. 11.

SUEYOSHI, Yuuji. Eine neue Methode zur Bestimmung von Phosphatid. 151.

SUGIYAMA, Goro. Beitrag zur Kenntnis der 3-Oxy-6-Ketoallocholansäure und Synthese der α-3,6-Dioxy-allo-Cholansäure. 157.

T

TAKAGI, Kenzo et HOTTA, Kazuo. Sur la teneur en cholestérine des ongles de quelques animaux. 109.

THOMAS, Karl und AKAO, Akira. Über α-Guanidoglutarsäure. Betrachtungen über die möglichen Vorstufen des Kreatins. 339.

Tissue, The micro determination of the iodine number of sterols in the ——s with special reference to ergosterol. 417.

TSUGE, Hidemaro. Studies in experimental scurvy. XXXV. On Bezssonoff's reacting substances, and on its identity to Vitamin C. 219.

TSUNOO, Shigeru. Über die Derivate der Aminooxypropansulfonsäure. Beiträge zur Kenntnis der Biuretreaktion. 375.

Trioxy-isosterolcholensäure, Konstitution der ——. 245.

Trypton, Über das Trypsinpepton ——. 329.

Tryptophan, Studien über den intermediären Stoffwechsel des ——s. 21.

U

UCHIMURA, Yukimaro. The effect of ultraviolet- and roentgen-rays on the redoxpotential of the frog muscle in vivo. 207.

Ultraviolet rays, The effect of —— —— and roentgen-rays on the redox-potential of the frog muscle in vivo. 207.

Uronic acid, On detoxication of naphthalene ingested, with special reference to a new —— —— excreted. 299.

V

van Slyke and Harington extraction chamber, A modification of the —— —— —— ——. 79.

Venous blood, The gaseous content of arterial, cutaneous and —— —— in the normal state, acidosis and alkalosis. 89.

Vitamin B_1, Chemistry of ——. 95.

Vitamin C, On Bezssonoff's reacting substances, and on its identity to ——. 219.

Y

YASUDA, Morio. The micro determination of the iodine number of sterols in the tissues with special reference to ergosterol. 417.

YOSHIKAWA, Haruhisa. Studien über die Bedeutung des Eisen-Porphyrins im Zellstoffwechsel. I. Eisen-Porphyringehalt und Atmungsfähigkeit der verschieden genährten Hefen. 627.

YOSHIMURA, Hisato and JUJIMOTO, Tomitaro. Is the hydrogen gas electrode not applicable to the determination of the pH of oxygenated blood? 493.

YOSHIMURA H., SHOJI, R., SAITO, K. and FUJIMOTO, T. Variations in the alveolar gaseous pressures as well as the gaseous contents of arterial blood and its pH in man at rest. 453.

YUSAWA, K. Sur le metabolisme de la cholestérine chez les cobayes inocules avec des bacilles tuberculeux. I. Sur le taux des cholestérine libres et éthérifiées dans le sang total du cobaye normal et du cobaye tuberculeux. 657.

YUUKI, Masaoki. Hagnesiumstoffwechsel und Gallensäure. Ausscheidung des Magnesium unter Einfluss von Cholsäure bei normalen sowie bei splanchnikotomierten Kaninchen. 435.

Z

Zellstoffwechsel, Studien über die Bedeutung des Eisen-Porphyrins im —— I. 627.

Zucker, Über den gärbaren, hydrolysierbaren —— im Blut, insbesondere dessen Mikrobestimmung. 1.